METHODS FOR THE INVESTIGATION OF STRUCTURE IN POLYPEPTIDES AND PROTEINS

A Thesis Submitted for the

Degree of Doctor of Philosophy

by

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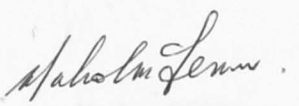
PREFACE

This thesis is submitted for the Degree of Doctor of Philosophy, having been submitted for no other degree. The work reported in this thesis was carried out in the Department of Chemistry of the Australian National University under the supervision of Dr. J.H. Bradbury. The work is wholly original except where due reference is made in the text.



(M.D. FENN)

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I would like to thank in general the staff of the Department of Chemistry and in particular my supervisor Dr. J.H. Bradbury for their encouragement and assistance during the course of the project. My thanks also go to Imperial Chemical Industries of Australia and New Zealand for their financial assistance in the form of an ICIANZ Research Fellowship.

Lastly, I express my thanks to my typist, Mrs. C. Grant, for persevering with a difficult task.

M.D. FENN

SYNOPSIS

This thesis is concerned with two aspects of structural peptide chemistry.

The first project reported in this thesis is an nmr study of the effect of strongly protic solvents on simple amides and a polypeptide, poly-γ-benzyl-L-glutamate (PBG). The results for simple amides are consistent with protonation on the addition of dichloroacetic acid (DCA) and relatively slow exchange of the amide nitrogen proton with solvent ($\tau > 10^{-2}$ sec).

Spin decoupling experiments conducted on solutions of PBG in protic and non protic solvents have shown that the greatest contributor to the width of the αCH resonance is due to coupling with the βCH_2 protons of the side chain. The exchange of the amide nitrogen proton with solvent was observed to be slow. A kinetic scheme for the helix to coil transition in PBG is proposed and a possible mechanism for the transition described.

The second project reported in this thesis involves the use of an inorganic complex, $Co\left[(triene)(OH)(OH_2)\right]^{++}$ as a sequenating agent. The complex has been reacted with both free peptides and C terminal protected peptides. The implications of C terminal protection is discussed. Optimal conditions for the stability of the intermediate peptide complex formed have been investigated and the rate of hydrolysis of this intermediate

complex to the amino acid complex and peptide less one residue determined. Both reaction rates were found to be dependent on the nature of the N terminal residue.

The possible use of an insoluble polymer support for the peptide in order to facilitate separation of reactants and products has been studied also.

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GENERAL INTRODUCTION

Very few problems in natural science have been subjected to such intensive investigation as the overall structure of proteins. One of the main features of protein investigations is the vast variety of experimental methods which have been used, from sophisticated techniques such as X-ray crystallography, nuclear magnetic resonance and electro optical methods to viscometry, osmotic pressure, electrical conductance, titration and simple organic chemistry. Such a multitude of techniques serves to illustrate the enormous complexity of not only the simplest living cell but also a native protein molecule. Proteins are considered to consist of three distinct structural groupings, aptly named the primary, secondary and tertiary structures (Linderstrom-Lang 1952). The primary structure can be defined as the actual chemical structure of the basic protein molecule. Secondary structure refers to stable configurations induced by hydrogen bonding of the primary structure. Tertiary structure describes the folding of this secondary structure in a compact molecular unit i.e. the overall shape of the protein molecule. The most intriguing problem presented to the protein chemist is, perhaps, the correlation of primary with secondary structure, secondary structure with tertiary and the three with function in proteins. This thesis consists of two projects, which at

first sight appear to be completely unrelated. However, in a broad sense, this is not quite correct. Both projects are concerned with a particular aspect of structural protein chemistry. The first is a continuation of project started previously (Fenn, 1967) and is concerned with an aspect of secondary structure, the transition between one secondary structure and another. The second project is concerned with the development of an inorganic technique for the determination of the primary structure in peptides and proteins.

NUCLEAR MAGNETIC RESONANCE THEORY

If an external magnetic field is applied to a system of nuclei with magnetic moments, these nuclear magnets will experience torques and will tend to be lined up parallel to the field. Although direct observation of such an orientation is difficult to observe, it is possible, under appropriate conditions, for these magnets to absorb energy from a magnetic field oscillating with a frequency in the radio frequency region. Such absorption gives rise to what are called nuclear magnetic resonance (nmr) spectra. Spectra are not observed for nuclei with no magnetic moment. Essentially, nmr spectra are used as a probe to investigate local magnetic effects inside a molecular system. The local magnetic field near a particular nucleus will depend on its chemical environment and is determined by several factors including the polarisation of remote parts of the molecule, magnetic moments (nuclear and electronic) of neighbouring molecules, and intra molecular effects due to other nuclei and electrons in the same molecule.

If a nucleus has a magnetic moment and a magnetic field is applied then the nucleus will precess in order to orientate its magnetic moment with the field. The energy required to do this is obtained from a discrete radio frequency

which is given by:-

$$\nu = \frac{\gamma Ho}{2\pi} \qquad (1)$$

where ν is the frequency in Hz

Ho the applied field

and γ the gyromagnetic ratio which is constant for each type of nucleus.

The second environmental effect is that of the surrounding electrons. If any atom or molecule is placed in a magnetic field it acquires by virtue of the induced orbital motion of its electrons a diamagnetic moment. These moving electrons constitute effective currents within the molecule and thereby produce secondary magnetic fields which also act on the nuclei present. Since the induced currents are proportional to the applied field, the magnitude of the induced field will also be proportional to the applied field, the local field at the nucleus being given by:-

$$H_{local} = Ho\ (1-\ \sigma\ ) \qquad (2)$$

where σ is a constant known as the screening constant dependent on the chemical environment of the nucleus. The effect of the screening constant can be seen from equation (1). For a finite positive value of σ the local field is lower and the frequency required for precession is lowered. For

the nmr spectra of a given species of nucleus in various chemical environments, there will be a corresponding set of different values of screening constants and resonance will occur at different frequencies for each chemically distinct environment. This displacement of signal due to the creation of screening constants is known as a chemical shift. When two or more nuclei have identical screening constants, identical chemical shifts are obtained and the nuclei are said to be equivalent.

In view of equations (1) and (2) the number of nuclei giving rise to each resonance is directly proportional to the intensity of the signal.

The most important single parameter to be derived from the nmr spectrum is the chemical shift. Since the absorption frequency of nuclei of different elements occur at very different parts of the spectra, normally only one type of nuclei is considered, in this case hydrogen. Since the absorption frequency is dependent on the applied magnetic field it is most convenient to express the chemical shift in terms of a non-dimensional unit defined by:-

$$\delta = \frac{H-H_s}{H_s} \qquad (3)$$

where H is the resonant frequency of the signal being measured and H_s is the resonant frequency of a second proton signal chosen as a reference signal.

The nmr spectrum may be modified if the molecules being measured are taking part in various rate processes, an example of which is the phenomenon of proton exchange between different chemical positions. If this exchange is rapid enough, the signals due to the protons at the various exchange sites will coalesce. Actually, this is merely the operation of the uncertainty principle, which in this case is given by equation (4):-

$$\tau = \frac{1}{2\pi\delta} \qquad (4)$$

where τ is the smallest lifetime for which the resonance of the two states can be distinguished and δ is the separation (in Hz) of the two resonances for large lifetimes. For lifetimes above this critical value, distinct resonances are obtained for protons at the exchanging sites and the intensity of the resonance is directly proportional to the number of protons at that exchange site. Furthermore, the width of the resonance is inversely proportional to the population.

i.e. $$\frac{W_a}{W_b} = \frac{P_b}{P_a} \qquad (5)$$

Where W_a and W_b are the widths of the resonances A and B respectively and P_a and P_b are their populations. Because of equation (5) when the population at one site is greatly in excess of the population at the other site, the resonance due to the latter site is broadened considerably and may become so broad as to be invisible in the spectrum (exchange broadening).

When the lifetimes are much smaller than the critical value, a single sharp resonance is observed. The observed chemical shift, δ_{obs} of the proton resonance is related to the chemical shifts of the various states by:-

$$\delta_{obs} = \sum P_i \delta_i \qquad (6)$$

where δ_i is the chemical shift of the protons at the i^{th} exchange site and P_i is the mole fraction of exchanging protons at this site. (Stewart et al, 1967).

When the lifetime of the exchanging protons is of the same order as the critical value, the resonances collapse to a single broad peak.

When all the magnetic nuclei in the molecule are equivalent then only one resonance signal is obtained. However, when the nuclei have two different sets of equivalent nuclei the spectrum may become considerably more complex. In

addition to the two distinguishable signals, each of these may be split into further components by spin-spin interaction. Due to interaction of the bonding electrons, two adjacent nuclei give rise to two different spin states (in the case of two adjacent protons):-

(a) where the nuclear spins are parallel;

(b) where the nuclear spins are anti parallel.

This gives rise to two slightly different magnetic states for each nuclei and hence two resonance frequencies for each nuclei. Since the difference in magnetic states is a function of the molecule it is independent of the strength of the applied field.

Spin-spin coupling may be effectively removed by several mechanisms which cause the spin multiplet to collapse to a less complex resonance.

The collapse of spin multiplets may be caused by the rapid exchange of nuclei between identical molecules. During exchange the exchanging nucleus has an equal probability of attaching itself to a molecule with any one of the spin orientations possible. If the exchange is rapid enough then the nuclei in the molecule do not see all possible spin states

of the exchanging nucleus, but an average and the spin multiplet collapses. The critical value for this process is again given by the uncertainty principle and the same observations for lifetimes greater than, smaller than or of the same order as the critical value apply.

Spin multiplets may also be collapsed by rapid disturbance or relaxation of the second nucleus causing the splitting. Such occurs with the resonance due to protons attached to a nitrogen nucleus. Under normal circumstances due to the three spin states of the nitrogen nucleus the resonance attributed to the proton is a triplet. When, as in certain molecules rapid transition of the nitrogen nucleus between its three spin states occurs, the proton sees only an average spin state and a single resonance ensues.

A third method is the application of a strong rf magnetic field, the frequency being adjusted to the resonance frequency of the nucleus causing the splitting. This causes frequent transitions between the various spin states of the nucleus, and just as with the preceding mechanism, an average spin state is seen and the spin-spin coupling is removed.

A final method of decoupling is to replace one of the coupling nuclei with a nucleus where the interaction of bonding electrons (hence splitting) is very small or negligible e.g. replacement of hydrogen by deuterium.

Hence by careful examination of the chemical shift, spin-spin splitting and width of the resonances, one may not only obtain information concerning the molecular structure of molecules but also the rates of any exchange processes which may be occurring.

SECTION 1:

NMR STUDY OF THE HELIX TO COIL TRANSITION IN PBG

INTRODUCTION

Proteins are naturally occurring high molecular weight compounds consisting of one or more chains of amino acid residues which are linked together by peptide bonds. The order in which the amino acid residues are arranged in the chain is known as the sequence (primary structure) and is unique for a particular protein. The chains may or may not contain inter or intra molecular crosslinks, and in many cases are associated with other compounds such as long chain fatty acids or sugars. Their molecular shapes vary from rigid rod like structures to the almost spherical symmetry of the globular proteins. Because of the great variation in the above factors the study and explanation of the physico-chemical properties of proteins has frequently proved to be difficult.

Understanding of these properties is often clarified by the study of simple 'protein like' model compounds. Although many small, low molecular weight peptides have been prepared by the well known step wise methods of synthesis and have proved useful models, the synthesis of reasonable quantities of high molecular weight polypeptides of a predetermined sequence is not yet technologically feasible. Thus there remains a large gap between the low molecular weight peptide models and the high

molecular weight proteins. This gap is bridged to some extent by the poly-α-amino acids.

Poly-α-amino acids are synthetic polymers of α-amino acids linked by peptide bonds, being prepared by the polymerisation of the corresponding N-carboxy acid anhydride monomer (Blout and Karlson, 1956, Katchalski et al 1964). Like other synthetic polymers a sample of poly-α-amino acid suffers from the disadvantage of being polydisperse i.e. the chain lengths of the molecules in the sample are of varying lengths.

The presence of the peptide bond and the relative ease with which poly-α-amino acids of a great variety may be synthesised make poly-α-amino acids very useful models for studying the physico-chemical properties of high molecular weight proteins in the solid state and in solution.

Physico-chemical properties of proteins which contain a large percentage of one amino acid may be approximated by a polymer of the amino acid concerned. For example poly-L-alanine has proved a useful model in the elucidation of the structure of tussah silk which contains a large amount of alanine. Similarly, polyglycine has been used as a model for silk fibroin and copolymers containing known sequences of glycine and proline for collagen. (Bamford et al, 1954).

Information pertinent to the primordial synthesis of proteins is obtained from the thermal polymerisation of mixtures of free amino acids, while kinetic studies of the polymerisation of N-carboxy acid anhydrides of amino acids may be useful in determining the probability of certain sequences occurring in proteins.

Multichain poly-α-amino acids exhibit the same physico-chemical properties as globular proteins and may prove useful models as such.

The information on poly-α-amino acids in the solid state has attracted the attention of a great number of investigators. Initially, the main purpose of this work was to arrive at a clearer interpretation of data obtained with fibrous and globular proteins and it soon became clear that poly-α-amino acids provide excellent model compounds for this purpose. X-ray analysis of poly-α-amino acids provided the first evidence for the existence of the α-helix conformation in proteins proposed by Pauling and Corey (1951). The work of Perutz (1951) and of Bamford et al (1952, 1956) showed that in the solid state poly-α-amino acids may attain α-helical conformations. It was, therefore, of interest to see whether such conformations existed in solution. The investigations carried out on the optical and hydrodynamic

properties, in particular those of Doty and coworkers (1954, 1956) on poly-γ-benzyl-L-glutamate have yielded evidence that poly-α-amino acids may exist in solution both in the αhelical form and random coil conformations, depending on the conditions chosen (e.g. solvent, temperature). It was found that in weakly interacting solvents such as chloroform, ethylene dichloride and dimethyl formamide poly-α-amino acids attain the helical conformation while in strongly interacting solvents (DCA, TFA) they exist in the form of random coils. Thus it is possible to induce a helix to coil transition by the gradual addition of a strongly interacting solvent to a solution of a poly-α-amino acid in a weakly interacting solvent. Studies on the helix-coil transition may shed some light on the process of protein denaturation which it appears to resemble in certain aspects. Although whether the transition is a good model for denaturation is doubtful since the rates for the two processes are vastly different (Bradbury and King, 1971).

The mechanism by which this helix to coil transition is achieved in such physical properties as viscosity (Doty et al, 1954, 1956; Teramoto et al, 1967; Bradbury and Fenn, 1967, Fenn, 1967; Bradbury and Fenn, 1968), specific volume

(Bradbury et al, 1965; Noguchi, 1966), heat capacitance (Ackermann and Ruterjans, 1964; Karasz et al, 1964; Karasz and O'Reilly, 1966; Ackermann and Neumann, 1967), optical rotatory dispersion (Doty and Yang, 1956; Yang and Doty, 1957; Perlmann and Katchalski, 1962; Moffitt and Yang, 1956; Fasman, 1962; Balusbramanian, 1967; Urnes and Doty, 1961; Quadrifoglio and Urry, 1967; Bovey, 1968; Marlborough et al, 1965) electrical birefringence (Watanabe et al, 1964; Milstein and Charney, 1970) and conductance (Stake and Klotz, 1965) has been a controversial subject for several years.

The effectiveness of these acids in producing transitions in the above physical properties has been attributed to their ability to disrupt the hydrogen bonds between the C=O and N-H groups which stabilise the helix, by forming their own competing hydrogen bonds with the C=O and N-H groups (Singer, 1962). However, it must be realised that DCA and TFA are strong acids and there exists the possibility of protonation of the amide group with subsequent collapse of the helix to the random coil, being brought about by electrostatic repulsion between charged amide groups.

Evidence purporting to show the validity of either one or the other of the above theories has been presented by many authors using a variety of techniques. Because of this apparent conflict of opinion it is proposed to summarise the present situation.

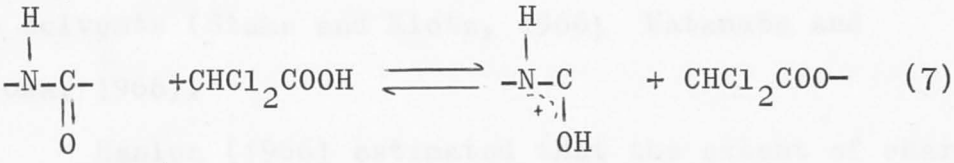
1. Model compounds

Specific volume, conductance, infra red and nmr studies of simple amides in concentrated aqueous solutions of mineral acids or in strongly protic solvents such as DCA or TFA have led to the conclusion that simple amides are indeed protonated in such solvents. (Berger et al, 1959; Herbison-Evans and Richards, 1962; Becker and Davidson, 1963; Klotz et al, 1964; Stewart et al, 1967; Nawrot and Veiss, 1970 (a)). Furthermore, it has been demonstrated that simple oligopeptides are protonated in formic acid, a much less protic solvent than either DCA or TFA (Nawrot and Veiss, 1970 (b)). Nmr studies of amides dissolved in a variety of protic and non protic solvents have demonstrated that when dissolved in DCA the resonance attributed to protons on the carbon adjacent to the amide nitrogen was approximately 0.24 ppm downfield from the position of the resonance in $CDCl_3$. This has been interpreted as being indicative of protonation (Fenn, 1967; Bradbury and Fenn, 1969 (a)). There is also evidence that protonation is preceded by a hydrogen bonded intermediate (Stewart et al, 1967; Nawrot and Veiss, 1970 (a)).

In brief, amides are protonated in DCA or TFA via a hydrogen bonded intermediate. On protonation a downfield shift of the resonance of the protons adjacent to the amide nitrogen occurs.

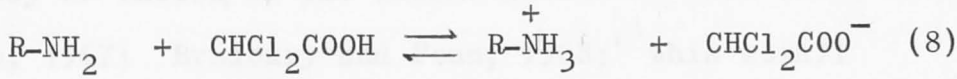
2. Poly-α-amino acids

Further direct evidence in favour of charging stems from the effect of the addition of amines, water (Steigman et al, 1969) and in particular the dichloroacetate ion (Zezine et al, 1968) to the solvent. Protonation of the poly-α-amino acid can be represented by the following equation.

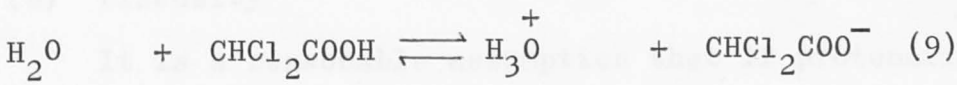


(7)

Whereas, the addition of amines or water results as shown by equations (8) and (9)



(8)



(9)

The addition of amines or water to the solvent will, as shown by equations (8) and (9), ~~will~~ increase the dichloroacetate ion. Hence, the equilibrium in equation (7) will be moved to the left. This results in the coil to helix transition which is observed.

(a) Infra red spectroscopy and conductivity

The possibility of protonation of the amide group of poly-α-amino acids was first proposed from infra red studies of several poly-α-amino acids in mixtures of non protic (EDC, $CHCl_3$) and protic (TFA, DCA) solvents. (Hanlon et al, 1963; Hanlon and Klotz, 1965; Hanlon, 1966). This proposal was substantiated by conductivity studies in the above solvents (Stake and Klotz, 1966; Watanabe and Yoshioka, 1966).

Hanlon (1966) estimated that the extent of charging of the amide groups of PBG in EDC-DCA mixtures in the region of 2-70% DCA was about 20% rising to 60%. This figure is greatly in excess of the amount estimated from other methods (Fenn, 1967; Bradbury and Fenn, 1968; this work).

(b) Viscosity

It is a reasonable assumption that if protonation were to occur when poly-α-amino acids are dissolved in organic acid solvents, then, presumably, one would find evidence of a polyelectrolyte effect in the viscometry.

The intrinsic viscosity, $[\eta]$, of a macromolecule is generally defined as $\underset{C=o}{Lt}\ \eta SP/C$ being obtained from the well known Huggins' equation

$$\eta_{SP}/_{C} = [\eta] + k [\eta]^2 C$$

by extrapolation of a graph of $\eta_{SP}/_{C}$ against C to zero concentration. For uncharged macromolecules whether rigid or flexible the above relationship has been found to be applicable. However, in the case of a charged macromolecule in the absence of a simple salt $\eta_{SP}/_{C}$ will increase with increasing dilution.

When a macromolecule is charged, due to repulsion between charges, there will be a relatively high free energy associated with a compact configuration and a relatively low free energy associated with an expanded one. For a high ionic strength the electrostatic free energy becomes considerably reduced by moderate expansion and the average configuration may be expected to continue to have spherical symmetry but with slightly increased radius of gyration. At low ionic strength, however, considerably more expansion is required. For a polyelectrolyte in solution in the absence of a simple salt, the only mobile ions present are the polymer counterions. On dilution with pure solvent the mobile counter ions will distribute themselves at a greater distance from the polyion. The concentration of these counter ions, and hence the ionic strength, will decrease in the vicinity of the polyion and the macromolecule will expand. Thus for increasing dilutions, increasing viscosities will be found:

a plot of η SP vs C having a characteristic negative slope.

Such an effect has been observed for poly-β-benzyl-L-aspartate, poly-ϵ-carbobenzoxy-L-lysine and PBG in DCA (Fenn, 1967; Bradbury and Fenn, 1968) poly-L-methionine in TFA (Bradbury and Chapman, 1970) and poly-L-lysine in concentrated H_2SO_4 (Peggion et al, 1970). It is also observed in 20% DCA/80% EDC for PBG samples of low degree of polymerisation (DPw $<$ 155). A similar polyelectrolyte effect has been noted for polyamides in formic acid (Schaefgen and Trivosonno, 1951, 1952; Saunders, 1962, 1964). Harrap and Woods (1961) observed a polyelectrolyte effect with proteins dissolved in formic acid. The effect was presumably due to protonation of side chain amines of the protein in this case.

An increase in flexibility of the helix on the initial addition of DCA to non protic solutions of high molecular weight poly-α-amino acids as shown by the decrease in the intrinsic viscosity and the decrease of the exponent in the Mark-Houwink equation is noted for PBG (Fenn, 1967; Teramoto et al, 1967; Bradbury and Fenn, 1968) and poly-γ-methyl-L-glutamate (Yoshida et al, 1962). It is worth noting that for high DP molecules where end effects are negligible the presence of one break in the helix lowers

the intrinsic viscosity by approximately 10% (see appendix A). As this is the order of magnitude of the observed decrease in $[\eta]$ one must assume that in the region 0-50% DCA/100-50% EDC only a very small number of internal breaks occur. The polyelectrolyte effect observed for small DP PBG in the solvent region associated with helix supporting properties has been interpreted as indicative of the presence of short, charged random coil breaks internally and at both ends of the helix. (Fenn, 1967; Bradbury and Fenn, 1968).

The amount of terminal disorder has been postulated to be independent of DP and small (10-20 residues at low acid concentrations). Hence, the effect from it will predominate at low DP's. On the other hand, the number of interruptions in the centre of the helix are proportional to the length of the chain but are still very few at high DP. (Fenn, 1967; Bradbury and Fenn, 1968). Thus the number of random residues will be virtually independent of DP, and any effect due to terminal disorder will only be observed for poly-α-amino acids of low DP.

The viscometry is considered to offer unequivocal proof of the presence of protonation of poly-α-amino acids in strongly protic solvents, since there is no other mechanism for the expansion of a macromolecule on dilution except if it be charged.

(c) Circular Dichroism and optical rotatory dispersion

Circular dichroism (Quadrifoglio and Urry, 1967) and optical rotatory dispersion studies (Balusbramanian, 1967; Perlmann and Katchalski 1962) on high DP poly- -amino acids in non-interacting solvents to which either TFA or DCA had been added showed no change in the various ORD or CD parameters until the normal helix to coil transition point was reached. Since viscometry (Fenn, 1967; Bradbury and Fenn, 1968) indicates that at low acid concentrations some 10-20 residues are in the random coil form, and hence protonated, at low molecular weights with, most probably, a slow increase with increase in molecular weight it is probable that in a high molecular weight sample these random coil residues will amount to a few per cent and any deviation in the CD and ORD parameters be within experimental error.

If this explanation is correct, then examination of a sample of PBG of low molecular weight should produce an appreciable effect. Such an effect has been observed by Fraser et al (1965) for a low molecular weight sample of poly-Y-ethyl-L-glutamate but not by Bovey (1968) for low molecular weight PBG.

D oxolupanine, a model amide, thought to be incapable of any structural change on protonation was shown by Bovey (1968) to have similar CD spectra in chloroform, trifluoroethanol, DCA and TFA. Bovey interpreted his results

as indicating the absence of protonation of the amide groups of D oxolupanine and hence the amide groups of poly-α-amino acids in the above solvents. However, Bovey assumed that the pKa's of the amide groups in D oxolupanine would be similar to those in poly-α-amino acids; an assumption which might well be incorrect. Neither did he offer any additional evidence i.e. conductivities to support his postulate of the absence of protonation.

Recent observations by Balusbramanian (1970) have indicated that the CD spectra of several poly-α-amino acids with non-ionisable side chains dissolved in a variety of non-protic helix disrupting media (fluoroketones and fluoroglycols) are similar to spectra obtained in concentrated sulphuric acid and methane sulphuric acid and again similar to spectra of poly-L-lysine and poly-L-glutamic acid in salt free aqueous media. In view of these observations there appears to be some doubt in the ability of CD and ORD spectra to distinguish uniquely between charged and uncharged species.

(d) Electro optical properties

Studies involving the measurement of electric dichroism, electric birefringence and dielectric constant of non-protic solutions of high molecular weight PBG to which DCA had been added have been made by several authors. (Watanabe and co-workers, 1964, 1966; Milstein and Charney, 1970).

It was observed that on the initial addition of DCA or TFA the electric birefringence and the dielectric constant drop to an observable plateau followed by an abrupt change accompanying the helix to coil transition in the vicinity of 75% DCA. Since electric birefringence can be related to the intrinsic viscosity (Tanford, 1961) and considering the high molecular weight of the PBG samples used, it is not surprising that the change in electric birefringence reflects the changes in intrinsic viscosity of a sample of PBG of similar molecular weight (Fenn, 1967; Bradbury and Fenn, 1968). However, the authors have interpreted the decrease in electric birefringence and dielectric constant as being due to a decrease in effective dipole moment. Watanabe has suggested the cause as being due to protonation of the terminal amide groups.

On the other hand, the addition of small amounts of TFA caused complete disappearance of electric dichroism. The authors (Milstein and Charney, 1970) interpreted this as being consistent with strong interaction of TFA with the benzyl ester carboxyls by hydrogen bonding. A similar effect has been observed on the addition of small amounts of DCA to $CDCl_3$ solutions of PBG (this work).

It can be said that the above studies have given some credulity to the postulate of protonation.

(e) Nuclear magnetic resonance spectroscopy

In recent years a great number of nmr investigations of the helix to coil transition of poly-α-amino acids in non aqueous solvents have been reported (Goodman and Masuda, 1964; Stewart et al 1967; Markley et al, 1967; Liu et al, 1967; Fenn, 1967; Bradbury et al, 1967, 1968; Bradbury and Fenn, 1969, (a), (b); Feretti and Paolillo, 1969; Feretti and Ninham, 1970; Liu and Lignowski, 1970). Although it is not proposed to discuss the experimental results of these investigations in detail, several basic observations can be made.

For poly-α-amino acids of high DP (> 100) the nmr resonance of the αCH proton is generally too broad to be observed in $CDCl_3$, but during the transition from the helix to the random coil occurs as a broad single peak which moves downfield. The presence of a single resonance has been interpreted as indicative of a magnetic environment for the αCH protons which is the average of that due to helical and random coil residues which are in rapid equilibrium. On the other hand with polypeptides of low DP (< 100) there are two resonances which can be attributed to the αCH protons. Such double resonances are a clear indication of two magnetic environments for the αCH which are in slow equilibrium.

Although these two peaks have been assigned to protonated and unprotonated residues (Fenn 1967; Bradbury and Fenn, 1969, (a), (b)) other authors (Feretti and Ninham, 1970; Bradbury et al, 1970; Ullman, 1970) have interpreted them differently.

It is the aim of this thesis to attempt to solve the apparent inconsistency between the spectra of high DP and low DP and the controversy over protonation of poly-α-amino acids.

EXPERIMENTAL

(a) Poly-α-amino acids

The preparation of all except one of the samples of PBG used have been described elsewhere (Fenn, 1967; Bradbury and Fenn, 1968). A sample of poly-γ benzyl-D-glutamate was prepared by a method previously described (Fenn, 1967). The number average degree of polymerisation of this sample as determined by n.m.r. end group analysis was 17 ± 2. Table I gives the degree of polymerisation of the various samples.

Table I

Sample No.	DP_w
MDF 11	13
MDF 18	17*
MDF 14	21
MDF 10	26
MDF 15	28
MDF 9	40

* DP_n Poly-γ-benzyl-D-glutamate

Deuterated samples of PBG were prepared by repeated lyophilisation from deutero TFA.

(b) Solvents

Deuterated Solvents

Deutero chloroform of spectroscopic quality was obtained from Stohler Isotope chemicals and used without further purification. Similarly, spectroscopic deutero trifluoroacetic acid (Merck) was used without further purification.

Deuterium oxide was obtained from the Australian Atomic Energy Commission.

Chloroform

Chloroform for nuclear magnetic resonance studies was of spectroscopic quality (Matheson, Coleman and Bell). The stabilising alcohol was removed by standing over Linde molecular sieve No 4A for two days, the chloroform was then decanted off and stored in a dark coloured bottle. Chloroform which had been stored for more than two weeks without the stabilising agent was discarded.

Dichloroacetic acid

B.D.H. laboratory grade acid was purified by distillation under reduced pressure at 1-2 mm pressure, B.P. 50-60^{o}C., the first 20% and the last 20% of the distillate being discarded. Experience has shown that the

middle fraction used contains between 0.1% and 0.3% water (Fenn, 1967).

Deuterated DCA was prepared by mixing quantities of DCA anhydride (B.D.H.) and D_2O in a dry box.

Trifluoroacetic acid

Light laboratory grade acid was dried over phosphorus pentoxide for several hours then distilled under anhydrous conditions (Klotz et al, 1964).

Other Solvents

The solvents, piperidine (M&B), pyrrolidine (Light), triethylamine (BDH) and benzene (M&B) were of laboratory grade quality and were used after drying over molecular sieve No 4A.

(c) Reagents

The following reagents were used without further purification.

Laboratory grade reagents

N methyl formamide (BDH) N methyl acetamide (BDH) diethyl acetamido malonate (Light) & NN dimethyl acetamide (K & K)

Analytical grade reagents

Potassium tertiary butoxide (M.S.A. Corp.)

(d) Nuclear Magnetic Resonance Methods

Spectra were obtained at 100mHz on a Varian HA100 instrument and at 60MHz on a Perkin Elmer R10 machine. Double resonance experiments were performed at 60MHz on a suitably modified Varian HA60 instrument by Dr. A.G. Moritz of the Defence Standards Laboratories, Melbourne (Long and Moritz, 1968). All spectra were obtained at 35°C using an r.f. input power of 1mV and a scanning speed of 1.6 ppm per minute. All solutions except where specifically stated were 10% w/v concentration. Mixed solvents were prepared by mixing accurately known volumes of the two components and are hence v/v%. Chemical shifts were measured with an accuracy of $\pm$ 0.02 ppm from an internal standard of tetramethylsilane and are recorded on the scale. Line widths represent the average of not less than three scans.

N-methyl acetamide $CH_3-CO-NH-CH_3$

Spectra were obtained in mixed solvents of $CDCl_3$ and DCA at 60MHz. The spectrum in $CDCl_3$ consists of a doublet at 7.28 tau and 7.20 tau (amide methyl) and a sharp singlet at 8.30 tau (aldehydic methyl). The resonance due to the NH proton was obscured by solvent. On deuteration of the amide by lyophilisation from D_2O, the doublet collapses to a singlet. Hence the splitting is due to spin-spin coupling with the amide hydrogen and not cis trans isomerisation. The addition of small amounts of DCA (9%) results in the collapse of the doublet to a broad singlet at 7.09 tau (Fig I). Additional acid results in little change in the spectrum. No additional broadening of the amide methyl is observed. The collapse of the doublet to a broad singlet indicates an increase in the rate of exchange of the amide proton with solvent protons. Clearly, this is an intermediate situation in which the lifetime of each state is of the same order as $2\frac{1}{2\pi\delta} \simeq 3 \times 10^{-2}$ sec where δ is the chemical shift between the two states and in this case is given by the coupling constant (J = 4.8 Hz). This lifetime is of the same order as that obtained by Stewart et al (1967) for N-methyl acetamide in 10% TFA - 90% $CDCl_3$. It is interesting to note that Stewart et al observed a decreased rate of exchange as shown by the

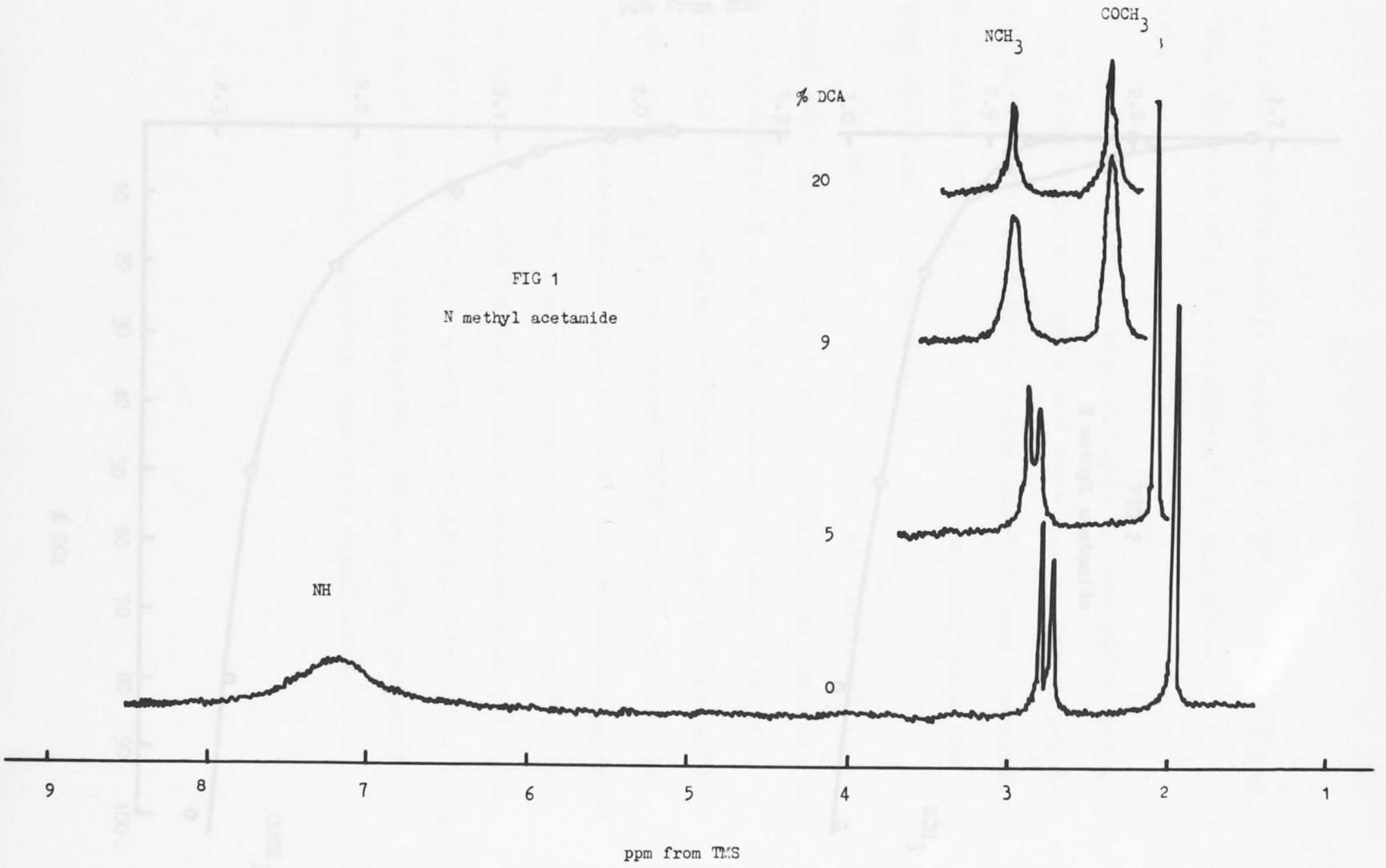


FIG 1

N methyl acetamide

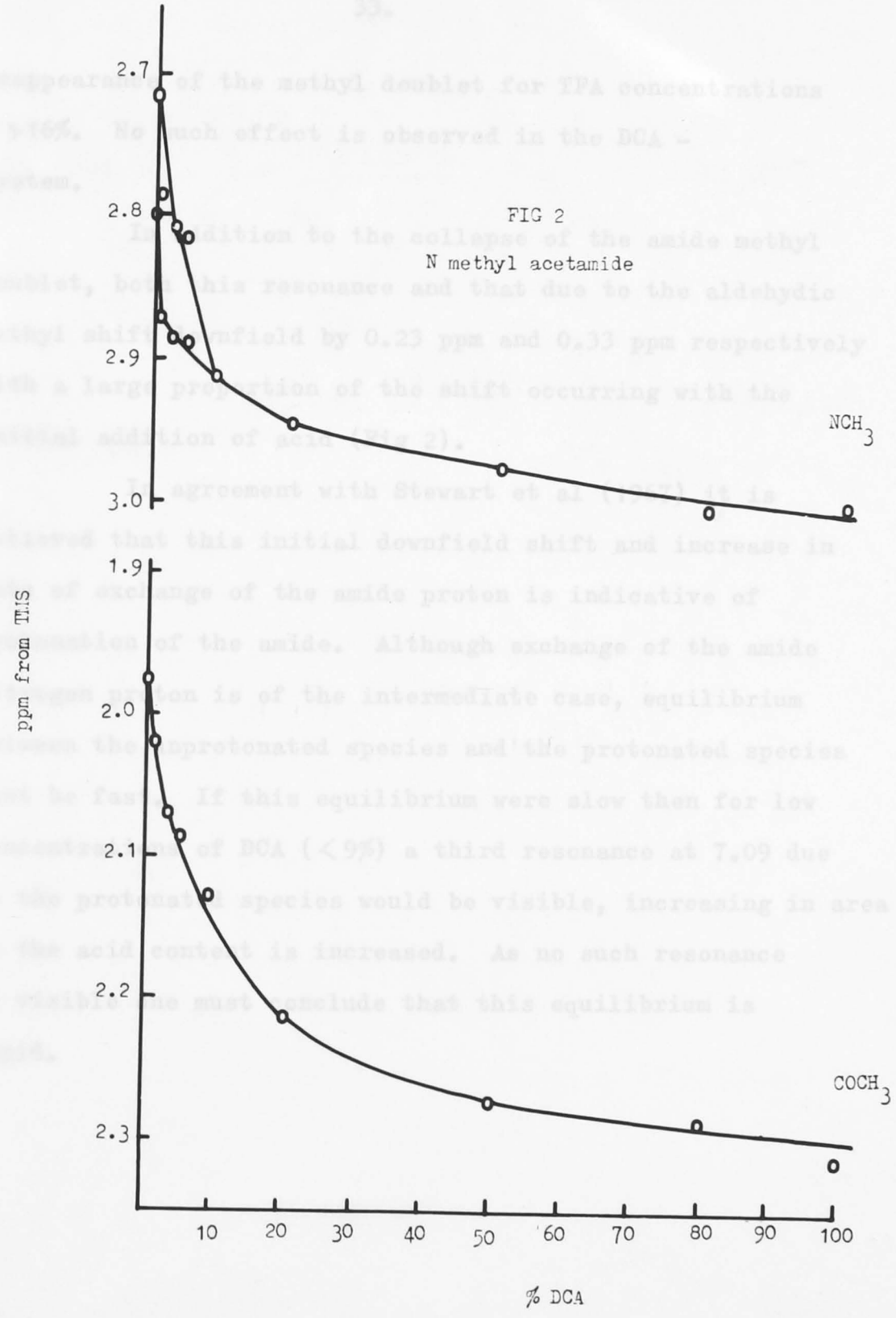


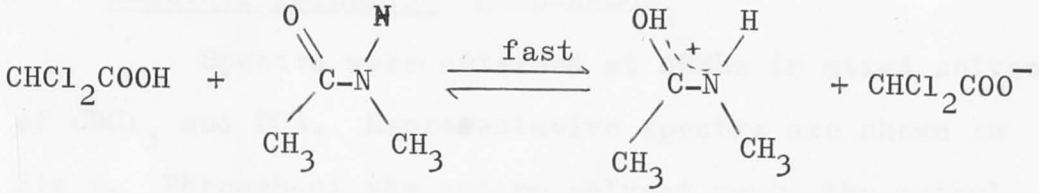
FIG 2

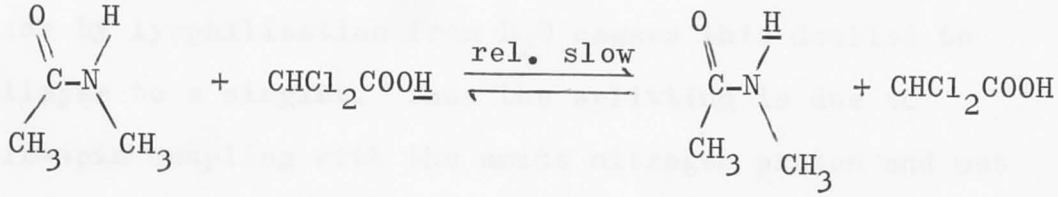
N methyl acetamide

reappearance of the methyl doublet for TFA concentrations >16%. No such effect is observed in the DCA - system.

In addition to the collapse of the amide methyl doublet, both this resonance and that due to the aldehydic methyl shift downfield by 0.23 ppm and 0.33 ppm respectively with a large proportion of the shift occurring with the initial addition of acid (Fig 2).

In agreement with Stewart et al (1967) it is believed that this initial downfield shift and increase in rate of exchange of the amide proton is indicative of protonation of the amide. Although exchange of the amide nitrogen proton is of the intermediate case, equilibrium between the unprotonated species and the protonated species must be fast. If this equilibrium were slow then for low concentrations of DCA (<9%) a third resonance at 7.09 due to the protonated species would be visible, increasing in area as the acid content is increased. As no such resonance is visible one must conclude that this equilibrium is rapid.


CHCl2COOH
O
C-N
CH3
CH3
fast
OH
H
+
C-N
CH3
CH3
+ CHCl2COO−


O
H
C-N
CH3
CH3
+ CHCl2COOH
rel. slow
O
H
C-N
CH3
CH3
+ CHCl2COOH

N-methyl formamide $H-CO-NH-CH_3$

Spectra were obtained at 60MHz in mixed solvents of $CDCl_3$ and DCA. Representative spectra are shown in Fig 3. Throughout the entire solvent range the methyl group appears as a doublet (J = 4Hz). Deuteration of the amide by lyophilisation from D_2O causes this doublet to collapse to a singlet. Thus the splitting is due to spin-spin coupling with the amide nitrogen proton and not cis trans isomerisation. The presence of the doublet also indicates that exchange between the amide proton and the solvent is slow i.e. $\tau = \frac{1}{2\pi\delta} \simeq 4 \times 10^{-2}$ sec. As the acid content of the solvent is increased both the methyl doublet and the broad singlet due to the aldehydic proton move downfield by 0.20 ppm and 0.15 ppm respectively (Fig 4). No broadening of these peaks outside of experimental error is observed.

The presence of one doublet throughout the solvent range indicates that the equilibrium between protonated and unprotonated species is fast.

On increasing the acid content of the solvent, the peak which can be attributed to the amide proton on area consideration, sharpens, moves downfield and increases in area (Figs 3, 5a). Only one resonance is observed for the amide

FIG 3

N methyl formamide

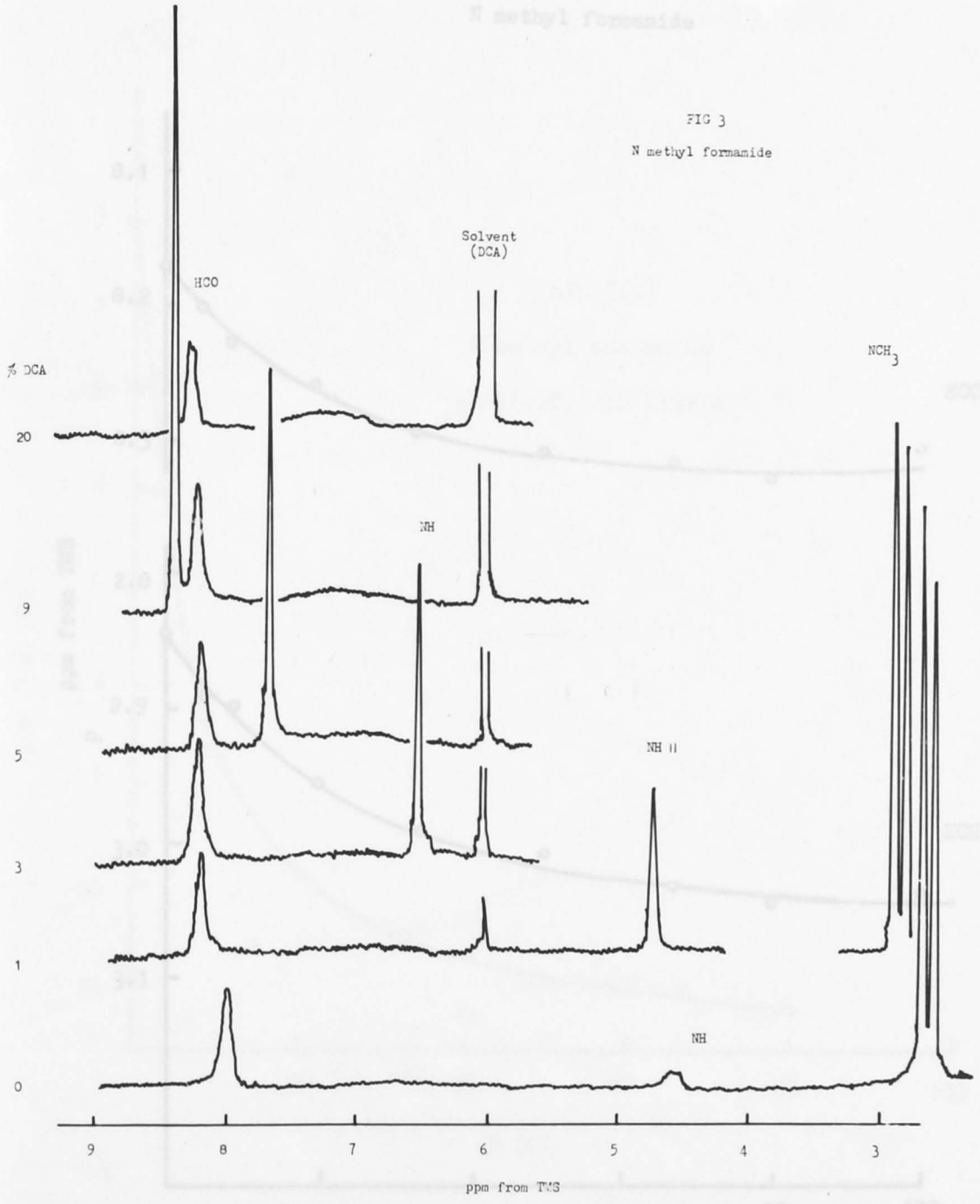
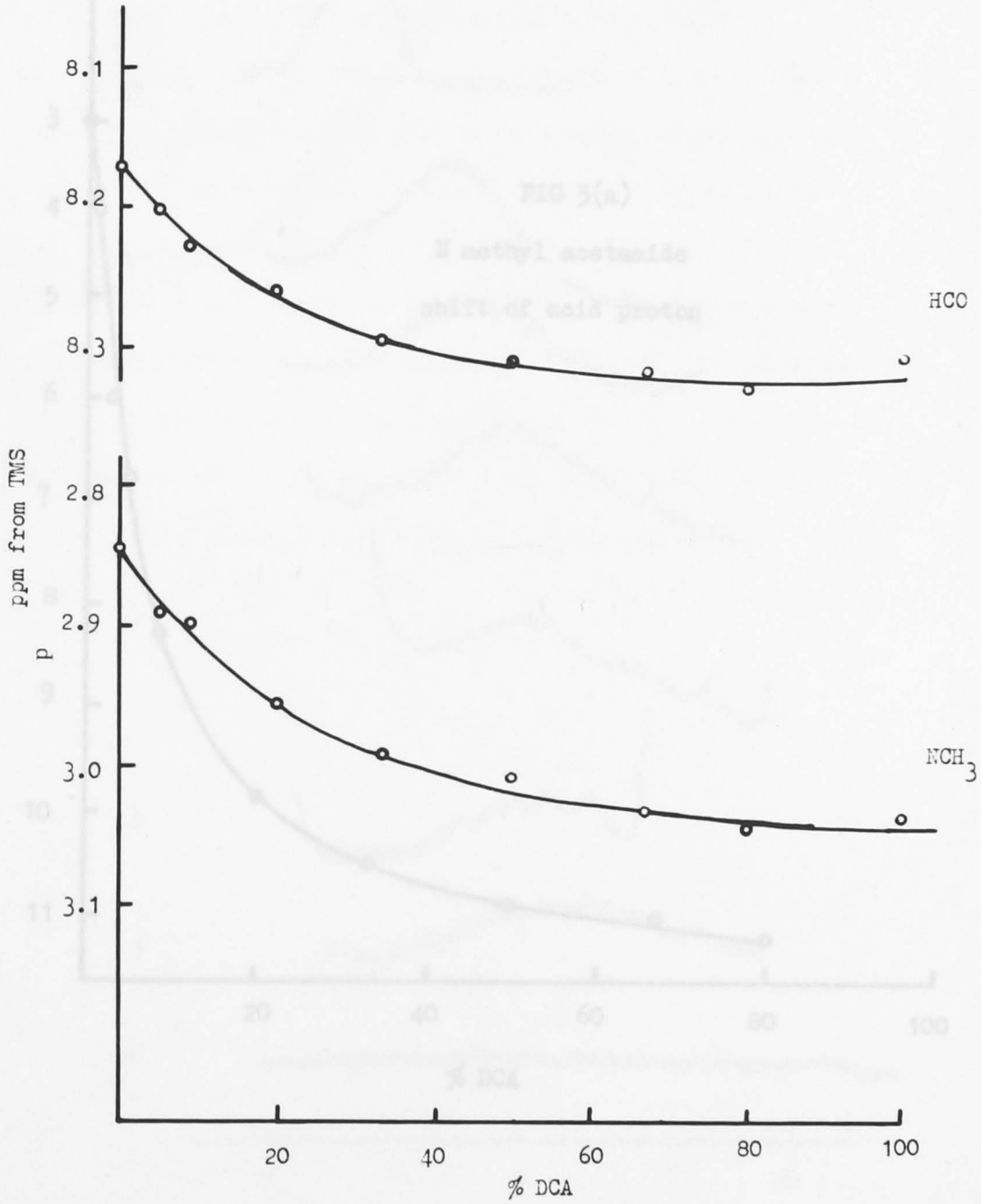


FIG 4

N methyl formamide



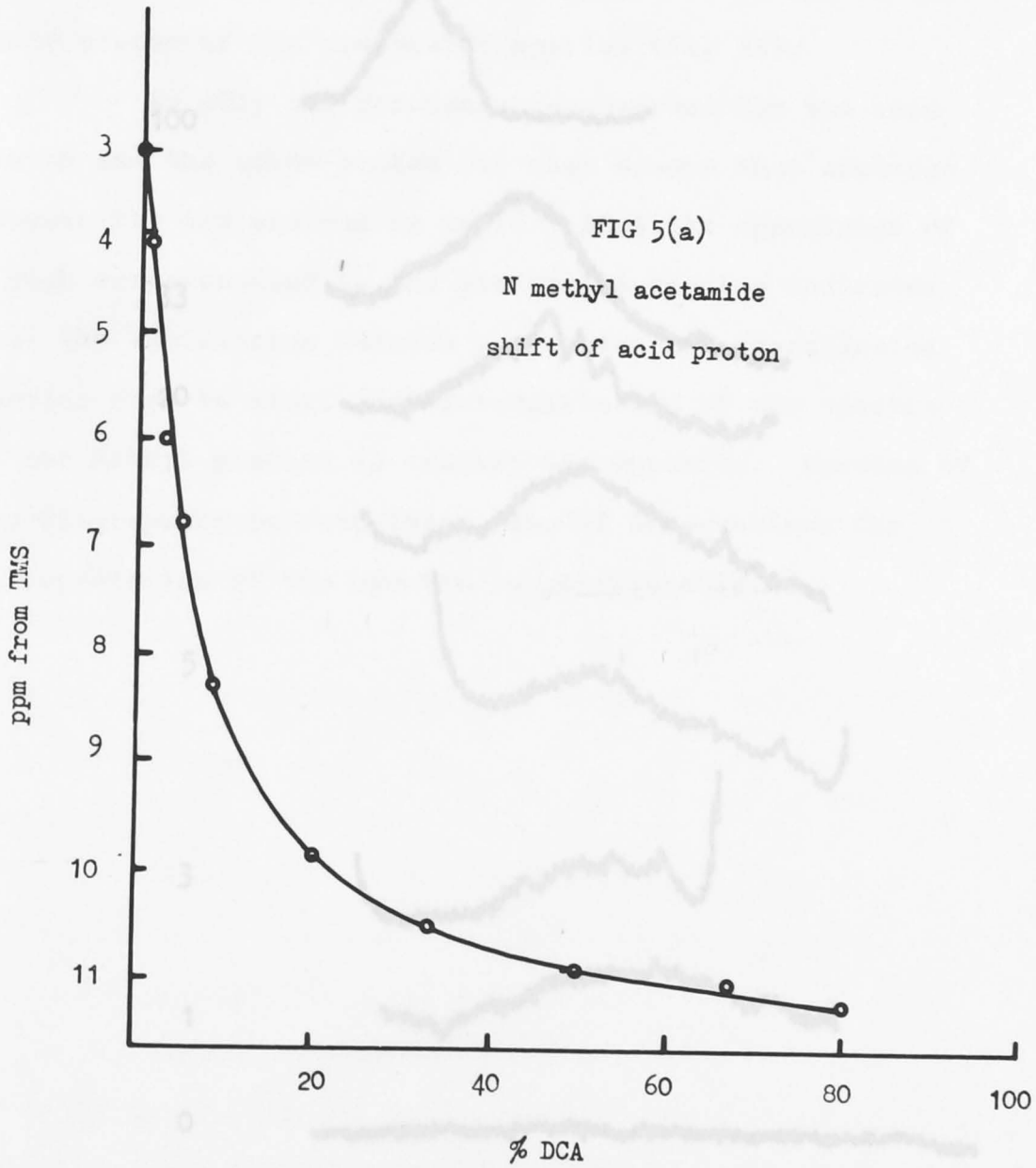
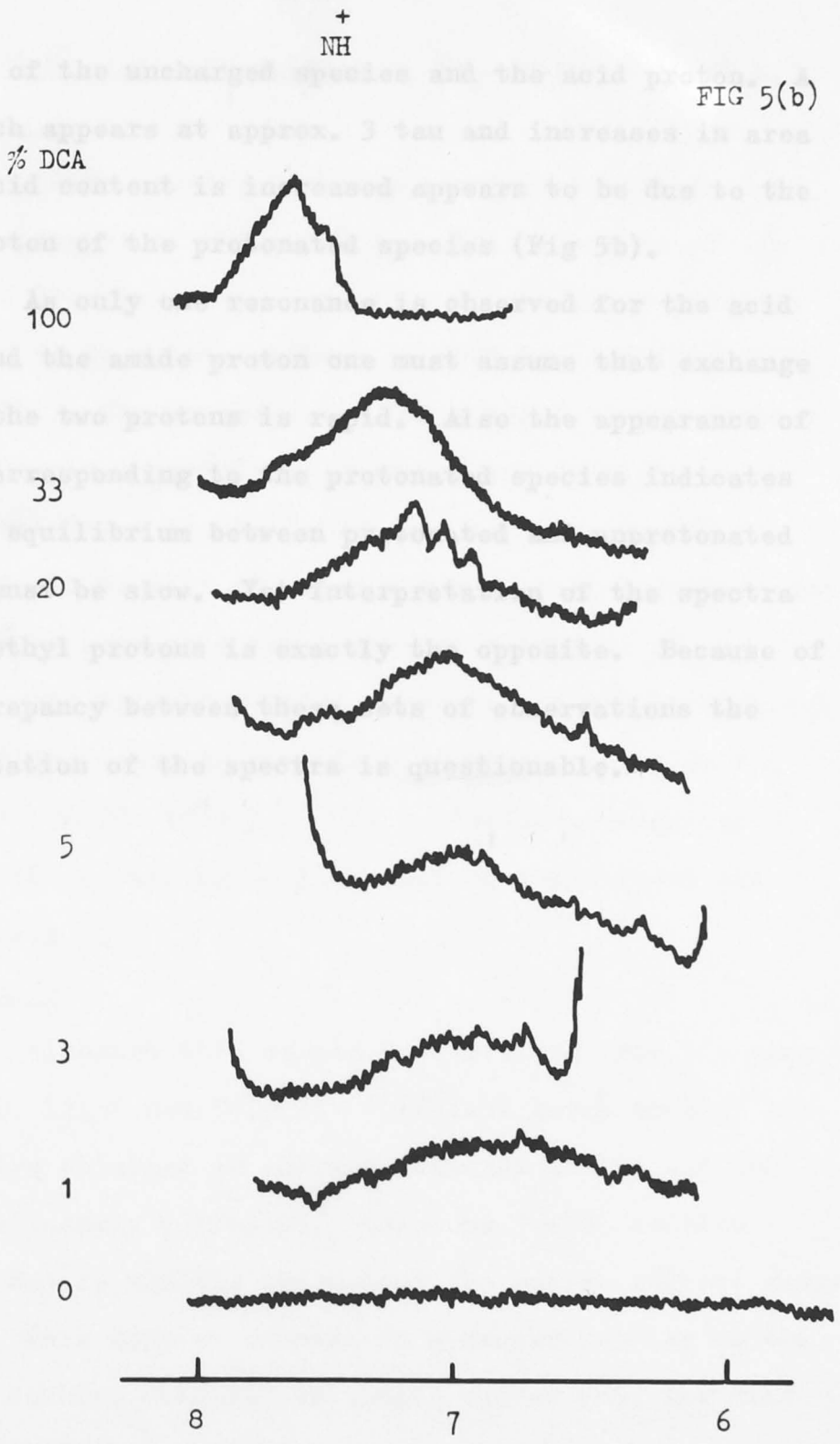


FIG 5(a)

N methyl acetamide

shift of acid proton

FIG 5(b)



hydrogen of the uncharged species and the acid proton. A peak which appears at approx. 3 tau and increases in area as the acid content is increased appears to be due to the amide proton of the protonated species (Fig 5b).

As only one resonance is observed for the acid proton and the amide proton one must assume that exchange between the two protons is rapid. Also the appearance of a peak corresponding to the protonated species indicates that the equilibrium between protonated and unprotonated species must be slow. Yet interpretation of the spectra of the methyl protons is exactly the opposite. Because of the discrepancy between these sets of observations the interpretation of the spectra is questionable.

Diethyl acetamide malonate $CH_3-CO-NH-CH-(COOC_2H_5)_2$

Sample No. 1

60MHz spectra for this sample were obtained in solvent mixtures of DCA and $CDCl_3$. The resonance of the proton on the carbon atom adjacent to the amide nitrogen was found to be a doublet both in 100% DCA and 100% $CDCl_3$ (J = 3.5 Hz), the resonance in DCA being 0.23 ppm further downfield. In intermediate solvents two doublets which can be attributed to the 'αCH' are visible (Fig 6). The downfield doublet observed moves rapidly downfield and increases in area with increasing acid content of the solvent. The upfield doublet shows only a slight chemical shift but decreases in area finally disappearing in the region 75-80% DCA (Fig 7). The variation in chemical shifts with increasing acid content of the solvent are shown in Fig 7.

Sample No. 2

Although this sample was obtained from the same source (L. Light and Co., but different batch number) the NMR spectra obtained in solvent mixtures of DCA and $CDCl_3$ are significantly different. Only one 'αCH' doublet (J = 3.5 Hz) is visible throughout the entire solvent range (Fig 8). This doublet behaves in a manner similar to the lowfield doublet observed in sample number one, the final chemical shift being identical.

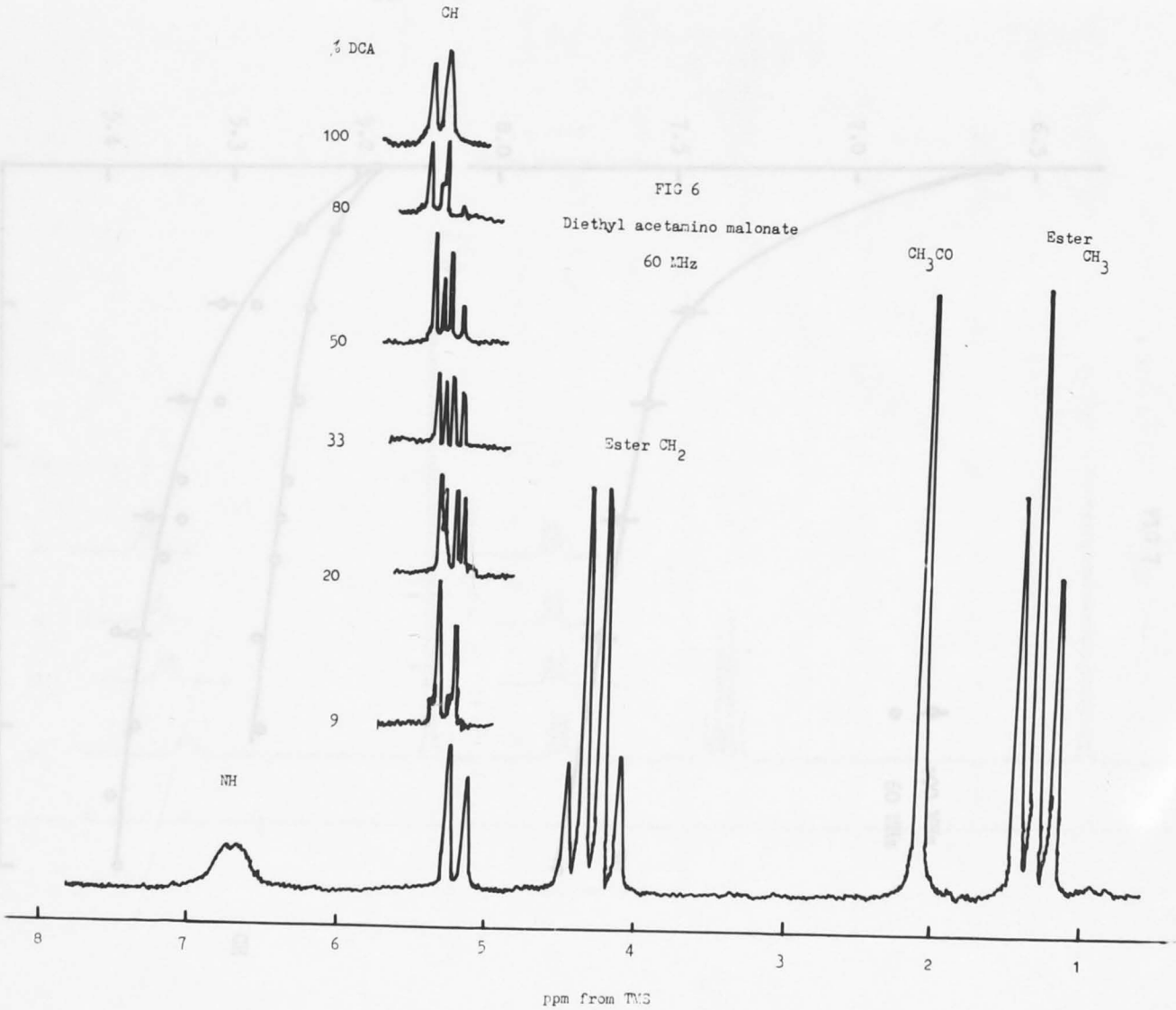
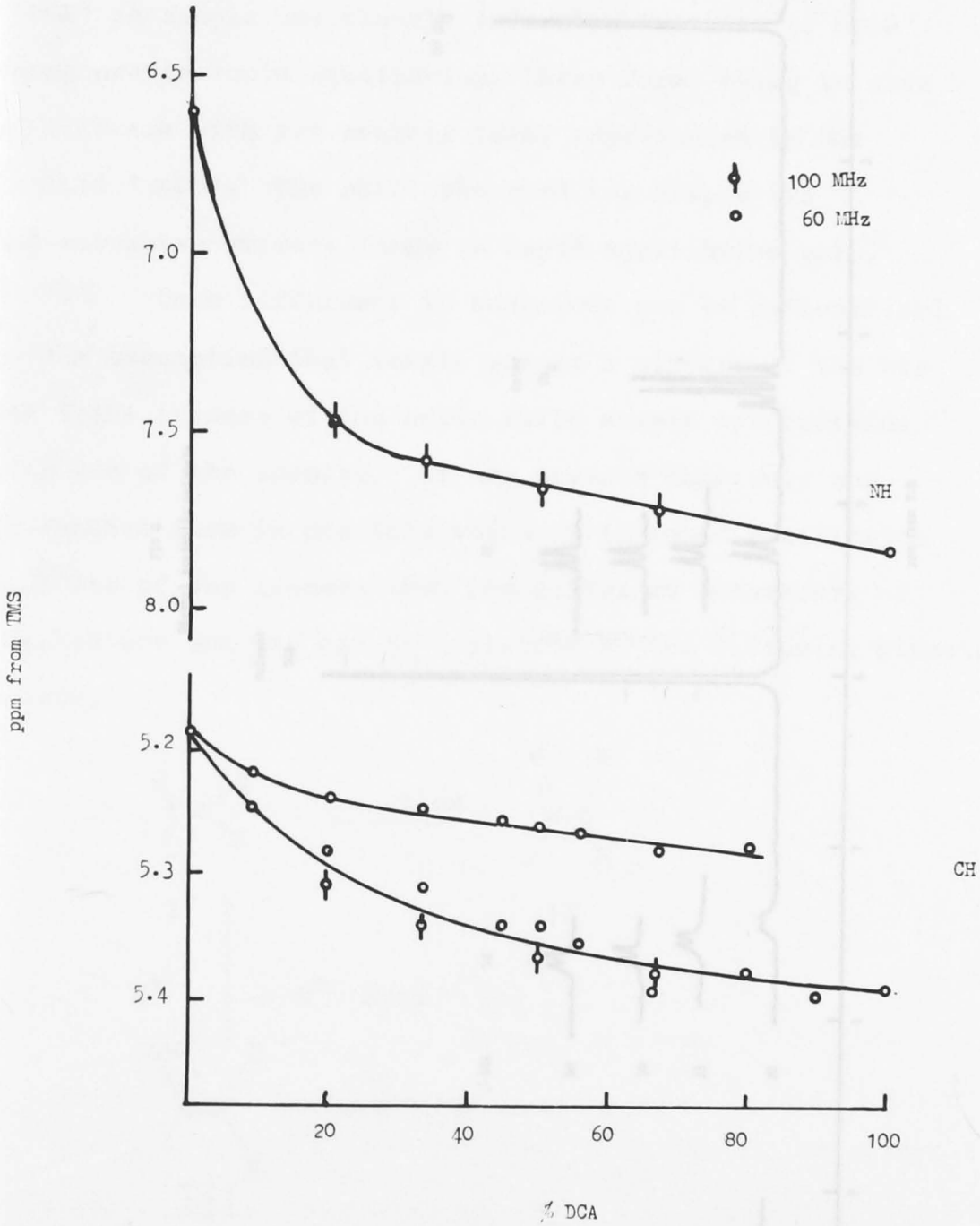


FIG 6

Diethyl acetamino malonate

60 MHz

FIG 7


6.5
7.0
7.5
8.0
100 MHz
60 MHz
NH
ppm from TMS
5.2
5.3
5.4
CH
20
40
60
80
100
% DCA

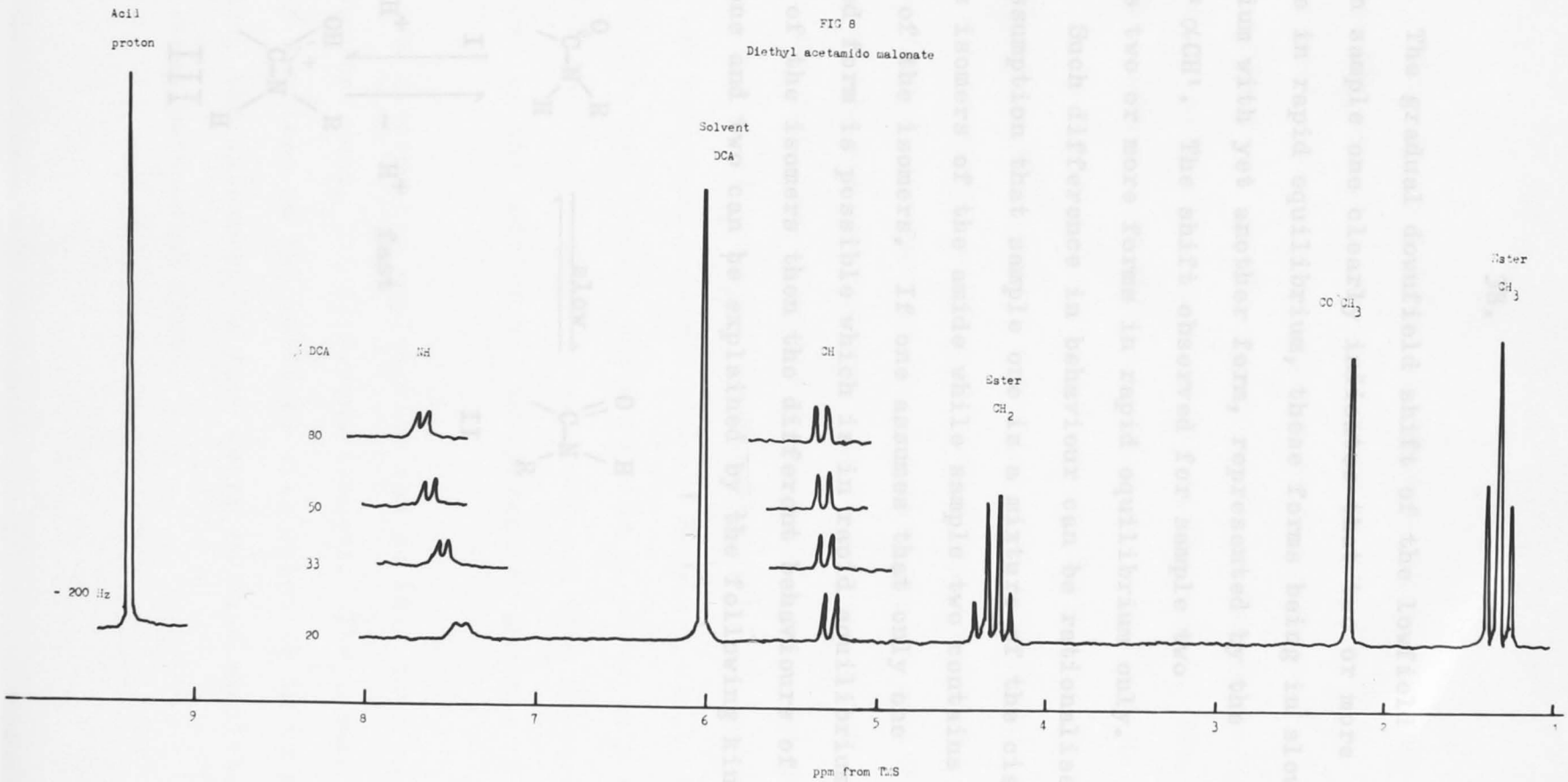
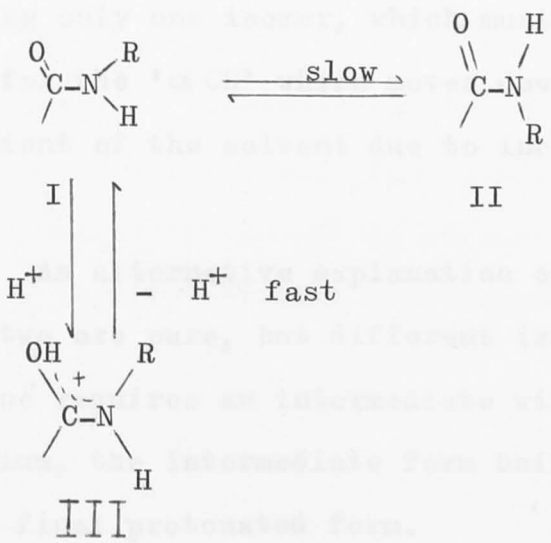


FIG 8

Diethyl acetamido malonate

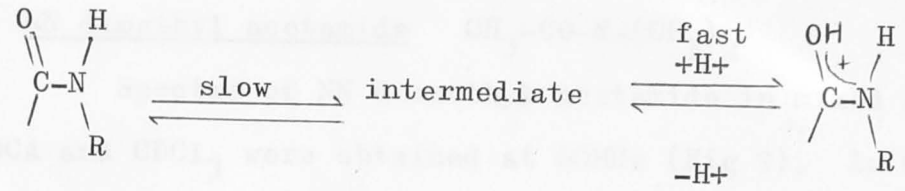
The gradual downfield shift of the lowfield 'αCH' in sample one clearly indicates that two or more forms are in rapid equilibrium, these forms being in slow equilibrium with yet another form, represented by the upfield 'αCH'. The shift observed for sample two indicates two or more forms in rapid equilibrium only.

Such difference in behaviour can be rationalised by the assumption that sample one is a mixture of the cis and trans isomers of the amide while sample two contains only one of the isomers. If one assumes that only one protonated form is possible which is in rapid equilibrium with one of the isomers then the different behaviours of samples one and two can be explained by the following kinetic scheme.

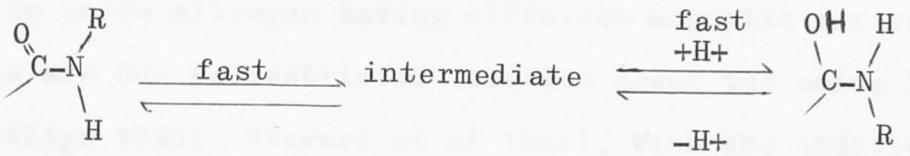


Since I and III are in rapid equilibrium with each other the observed chemical shift of the 'αCH' resonance will represent an average of the relative amounts of the two. As the acid content of the solvent is increased the relative amount of III is increased. Since the 'αCH' resonance of the protonated amide III can be expected to be further downfield than in II (Bradbury and Fenn 1969, (a)), the average resonance will move downfield. As II is not in rapid equilibrium with I two resonances will be seen. With an increase in the relative amount of III more of I is removed and the equilibrium between I and II shifts to the left thus decreasing the relative amount and hence the area of II. Sample one containing both isomers exhibits the behaviour outlined above. However, sample two containing only one isomer, which must be I, shows only one doublet for the 'αCH' which moves downfield with increasing acid content of the solvent due to increasing amounts of III.

An alternative explanation can be that both samples one and two are pure, but different isomers. The isomer in sample one requires an intermediate with which it is in slow equilibrium, the intermediate form being in fast equilibrium with the final protonated form.



While the isomer in sample two either requires no intermediate or has an intermediate which is in rapid equilibrium with both the isomer and the final protonated form.



Although the equilibrium between protonated and unprotonated species appears to be rapid, the rate of exchange of the amide proton with solvent appears to be slow as shown by the presence of a doublet for the 'αCH' resonance (J = 3.5Hz). Also a separate doublet for the amide proton (J = 3.5Hz) distinct from the acid proton is observed (Fig 8). This resonance moves downfield as the acid content of the solvent is increased again signifying fast equilibrium between the protonated and unprotonated forms. (It is noted that the interpretation of the chemical shifts of all resonances lead to the same conclusions, a fact not observed in the case of N methyl formamide.)

NN dimethyl acetamide $CH_3-CO-N-(CH_3)_2$

Spectra of NN diemethyl acetamide in mixed solvents of DCA and $CDCl_3$ were obtained at 60MHz (Fig 9). In $CDCl_3$ the spectrum consists of a doublet at 6.98 and 7.07 tau (amide nitrogen methyls) and a singlet at 7.93 tau (aldehydic methyl). The doublet is attributed to the two methyl groups on the amide nitrogen having different magnetic environments which are due to restricted rotation about the amide bond (Phillips 1955; Stewart et al 1967). With the addition of DCA to the solvent (9%), the doublet collapses to a singlet indicating an increase in the rate of rotation about the amide bond to such an extent that the different magnetic environments of the methyl groups are averaged out and a single resonance is observed. Similar behaviour was observed by Stewart et al in a TFA/$CDCl_3$ system. Although in the TFA/$CDCl_3$ system the doublet collapsed at a slightly higher concentration of acid (12%) than in the DCA/$CDCl_3$ system used here. In an agreement with other authors (Stewart et al) it is felt that the collapse of the doublet is due to protonation of the amide group with consequent loss of double bond character of the CO-N bond. Such acid catalysed lowering of the rotational barrier has also been observed in aqueous hydrochloric acid solutions (Berger et al 1959). On protonation both the

FIG 9

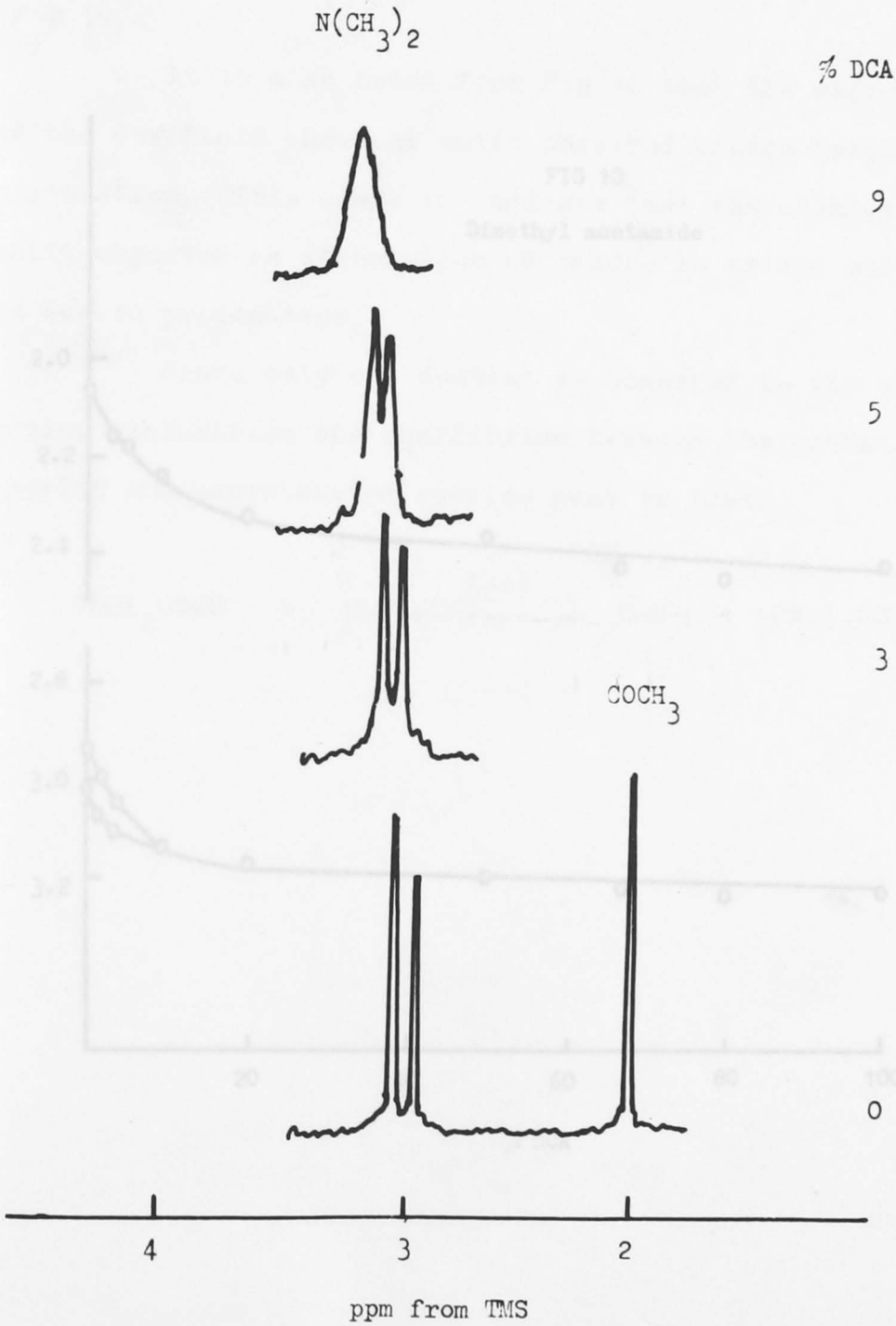
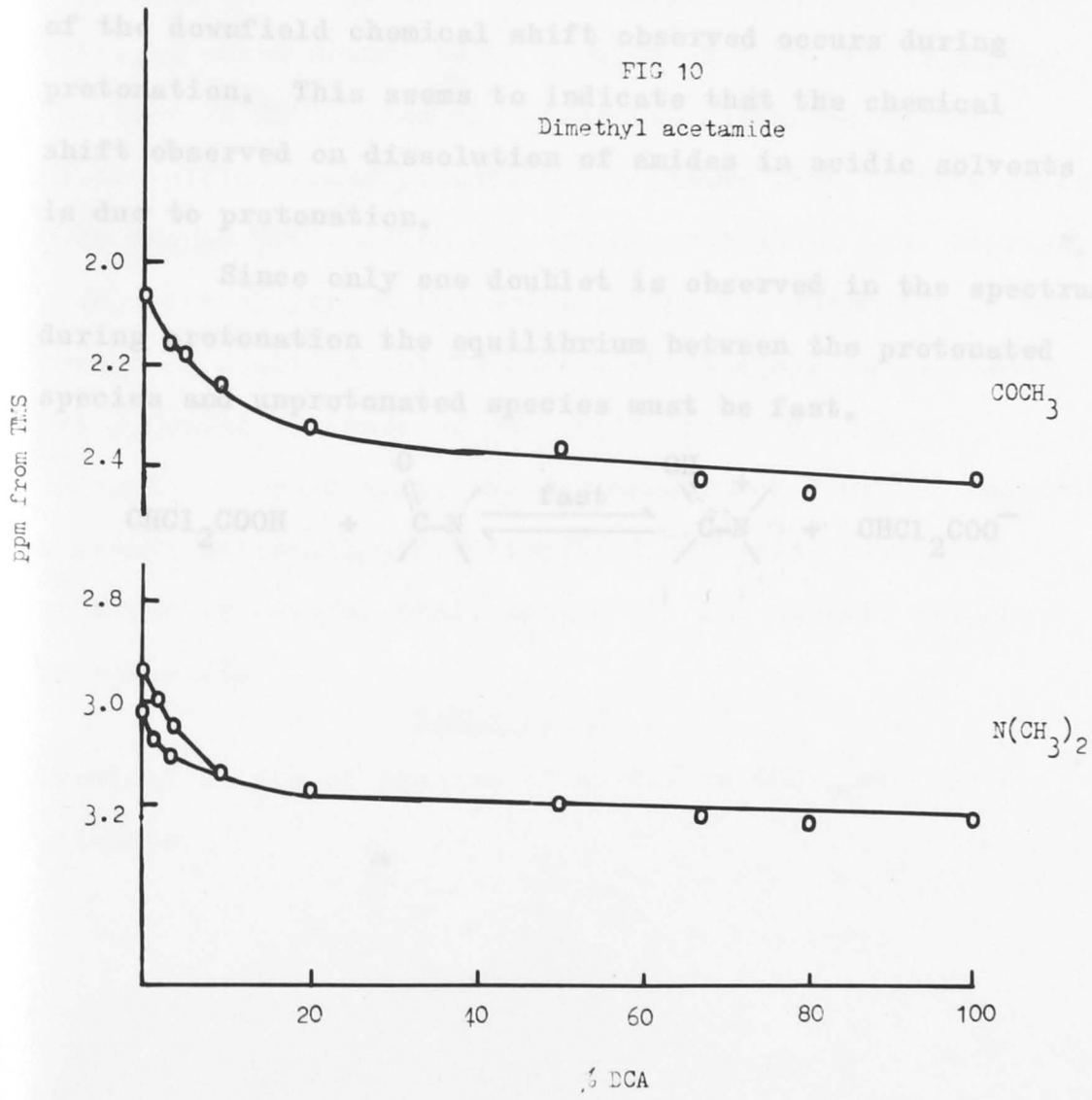


FIG 10

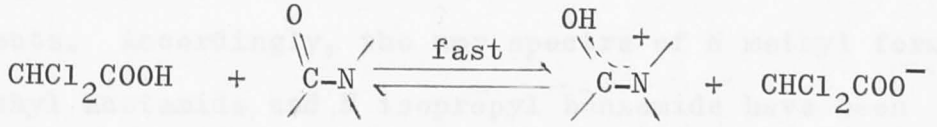
Dimethyl acetamide



aldehydic methyl group and the amide methyl resonances move downfield by 0.30 ppm and 0.24 ppm respectively (Fig 10).

It is also noted from Fig 10 that the majority of the downfield chemical shift observed occurs during protonation. This seems to indicate that the chemical shift observed on dissolution of amides in acidic solvents is due to protonation.

Since only one doublet is observed in the spectrum during protonation the equilibrium between the protonated species and unprotonated species must be fast.



NMR study of simple amides in basic solvents

Previous work (Fenn, 1967; Bradbury and Fenn, 1969, (a)) had shown that for a simple amide the n.m.r. resonance of the protons on the carbon atom adjacent to the nitrogen moved downfield by a value of 0.24 ppm from its position in $CDCl_3$ when the amide was dissolved in DCA. It therefore seemed reasonable to assume that if this shift were due to protonation then on deprotonation i.e. placing, a negative charge on the amide group, the resonance of the proton in question should have a shift equal in magnitude but opposite in sense to the shift observed in acid solvents. Accordingly, the nmr spectra of N methyl formamide, N methyl acetamide and N isopropyl benzamide have been obtained in several basic solvents. The results are shown in table II.

TABLE II

Chemical shifts of protons of amides in $CDCl_3$ and basic solvents.

Amide	$CDCl_3$ - solvent (ppm) in			
	Et_3N	Piperidine	Na/C_6H_6	$Bu^tOK/DMSo-d_6$
$HCONH-\underline{CH_3}$	0	a	-0.03	-0.22
$CH_3CONH-\underline{CH_3}$	0	a	-0.05	-0.23
$PhCONH-\underline{CH}(CH_3)_2$	0	-0.03	-0.07	-0.24

a obscured by solvent

Triethylamine and piperidine are not strong enough bases to cause a chemical shift due to deprotonation, however, sodium in benzene has a small effect. Apart from this the spectra are essentially unchanged in these solvents and the NH resonance is visible. When equimolar amounts of the amide and potassium tertiary butoxide are dissolved in dimethyl sulphoxide-d_6 the following spectral changes are observed:-

(a) a decrease in the amount of splitting of the proton resonance adjacent to the amide group and a chemical shift of 0.23 ppm which is equal in magnitude but opposite in sense to the average shift of 0.24 ppm observed on dissolution in DCA (Fenn 1967, Bradbury and Fenn 1968);

(b) the NH resonance disappears;

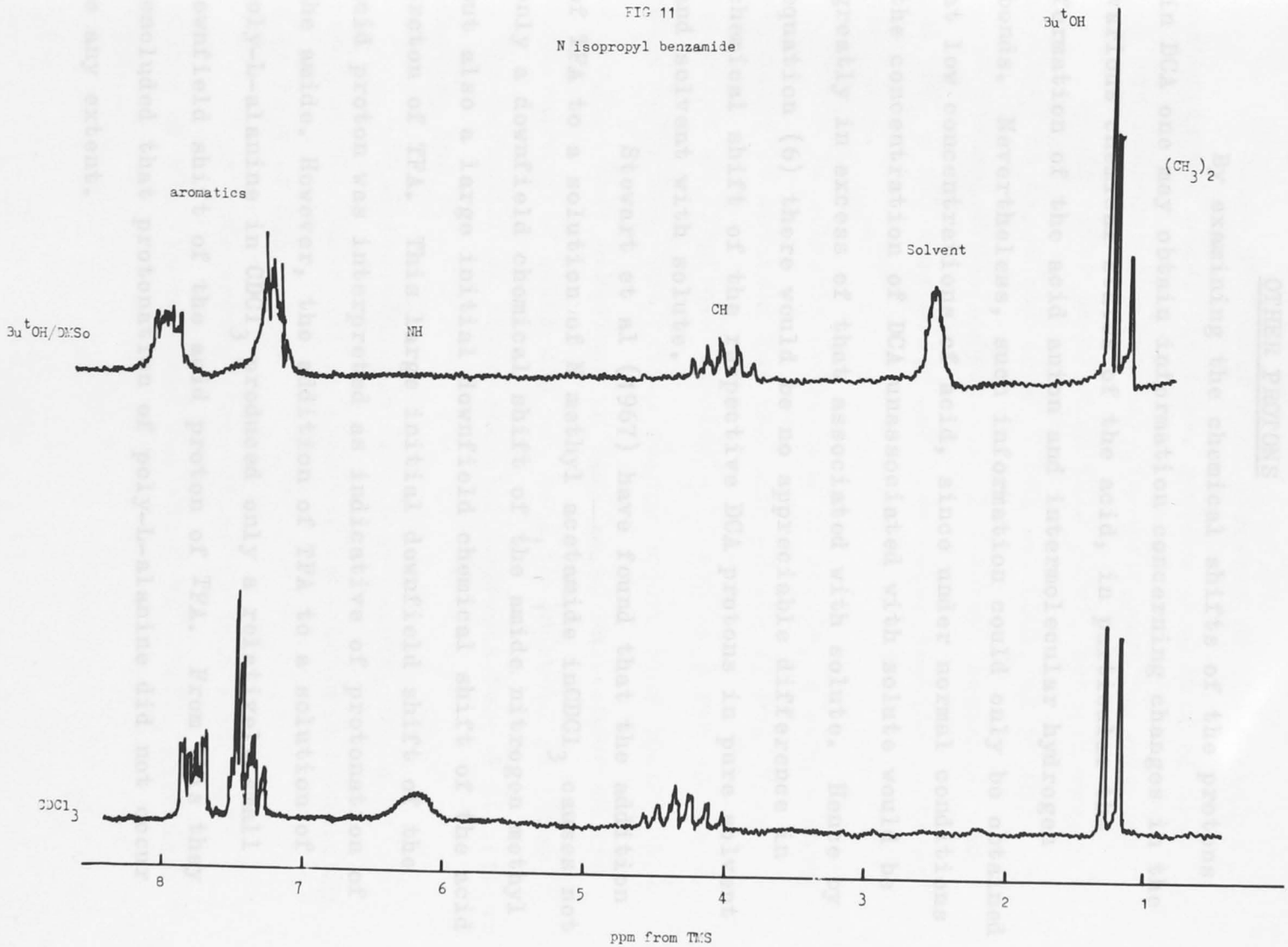
(c) the occurrence of a new resonance at 8.72 due to the methyl groups of tertiary butanol which is produced by deprotonation of the amide group.

Fig. 11 illustrates the above being the spectra of N isopropyl benzamide in $CDCl_3$ and $Bu^tOK/DMSo\text{-}d_6$.

It is also observed from the table that since N isopropyl benzamide is the most difficult to protonate (Fenn 1967; Bradbury and Fenn 1969 (a)) it is conversly the easiest to deprotonate.

FIG 11

N isopropyl benzamide



OTHER PROTONS

By examining the chemical shifts of the protons in DCA one may obtain information concerning changes in the various chemical states of the acid, in particular the formation of the acid anion and intermolecular hydrogen bonds. Nevertheless, such information could only be obtained at low concentrations of acid, since under normal conditions the concentration of DCA unassociated with solute would be greatly in excess of that associated with solute. Hence by equation (6) there would be no appreciable difference in chemical shift of the respective DCA protons in pure solvent and solvent with solute.

Stewart et al (1967) have found that the addition of TFA to a solution of N methyl acetamide in $CDCl_3$ causes not only a downfield chemical shift of the amide nitrogen methyl but also a large initial downfield chemical shift of the acid proton of TFA. This large initial downfield shift of the acid proton was interpreted as indicative of protonation of the amide. However, the addition of TFA to a solution of poly-L-alanine in $CDCl_3$ produced only a relatively small downfield shift of the acid proton of TFA. From this they concluded that protonation of poly-L-alanine did not occur to any extent.

Since the molar concentration of the N methyl acetamide was about six times greater than that of the peptide residues the two experiments cannot be compared.

In order to clarify this point, spectra were obtained in mixed solvents of DCA and $CDCl_3$ at 60 MHz where the molar concentrations of N methyl acetamide and PBG (DP_w = 21) were 0.44M and 0.46 moles peptide residues per litre.

In solvents containing no solute, a single sharp resonance is obtained for the acid proton of DCA. This resonance is displaced downfield by nearly 3 ppm in the range 1-30% DCA, but little change occurs from 30-100% DCA (Fig 12). This behaviour is similar to that observed for other carboxylic acids and has been interpreted in terms of the formation of intermolecular hydrogen bonded structures (Reeves, 1961).

With the addition of either solute, the acid proton of DCA could not be observed in solutions containing 5% or less of DCA.

An interesting observation is that when small amounts of DCA (5% or less) are added to $CDCl_3$ solutions of PBG:-

FIG 12

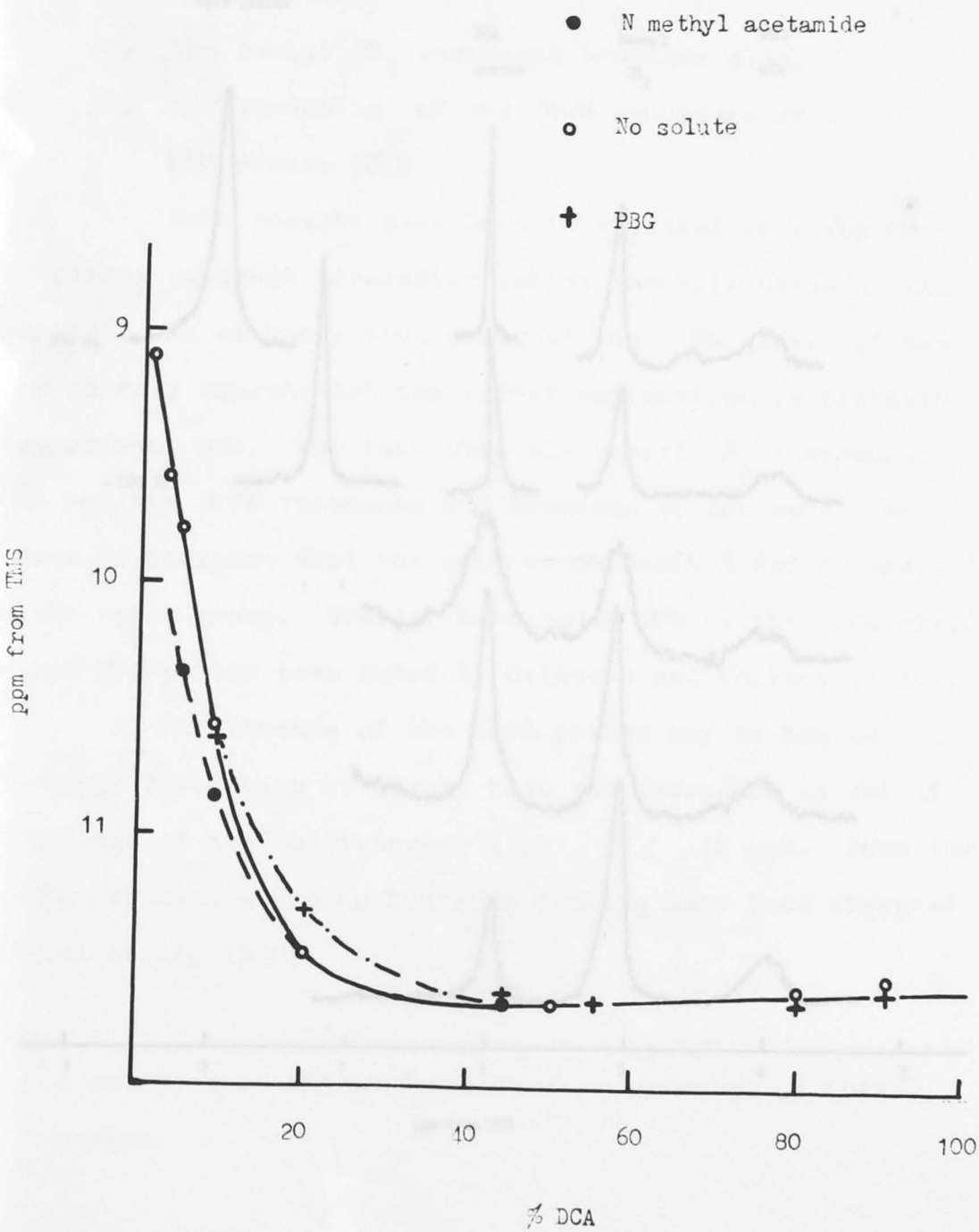
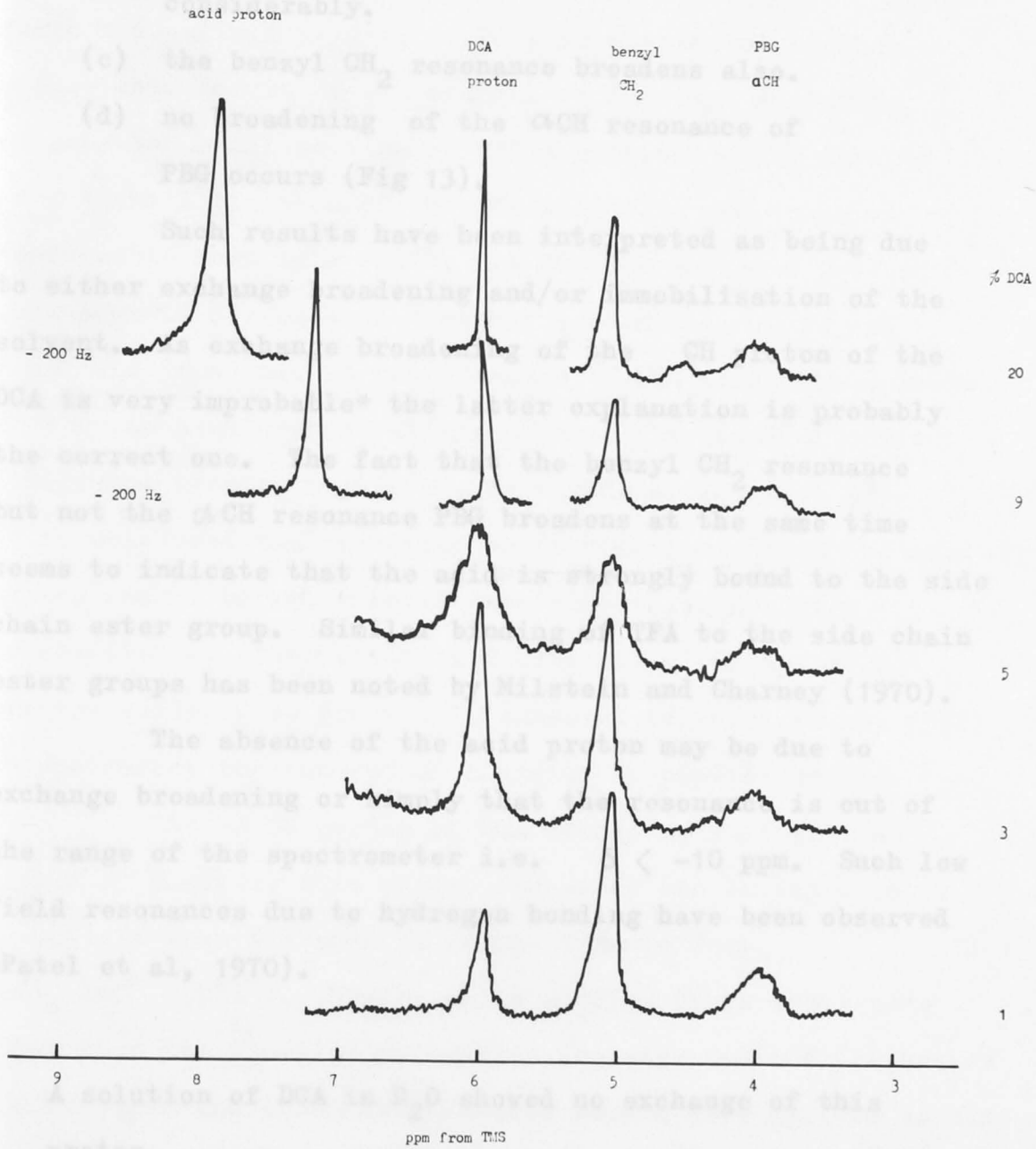

N methyl acetamide
No solute
PBG
9
10
11
ppm from TMS
20
40
60
80
100
% DCA

FIG 13


acid proton
DCA
proton
benzyl
CH_2
PBG
αCH
% DCA
- 200 Hz
- 200 Hz
20
9
5
3
1
9
8
7
6
5
4
3
ppm from TMS

(a) the acid proton resonance cannot be seen.

(b) the CH resonance of the acid broadens considerably.

(c) the benzyl CH_2 resonance broadens also.

(d) no broadening of the αCH resonance of PBG occurs (Fig 13).

Such results have been interpreted as being due to either exchange broadening and/or immobilisation of the solvent. As exchange broadening of the CH proton of the DCA is very improbable* the latter explanation is probably the correct one. The fact that the benzyl CH_2 resonance but not the αCH resonance PBG broadens at the same time seems to indicate that the acid is strongly bound to the side chain ester group. Similar binding of TFA to the side chain ester groups has been noted by Milstein and Charney (1970).

The absence of the acid proton may be due to exchange broadening or simply that the resonance is out of the range of the spectrometer i.e. $\delta < -10$ ppm. Such low field resonances due to hydrogen bonding have been observed (Patel et al, 1970).

* A solution of DCA in D_2O showed no exchange of this proton.

At 10% DCA the acid proton resonance in the solvent mixture alone and in the presence of PBG occurs at 0.50 whilst that in the presence of N methyl acetamide was 0.50 ppm further downfield (Fig 12). This is attributed to the appreciable amount of protonation of N methyl acetamide, whereas the small portion of PBG protonated would involve only a small fraction of the DCA molecules and cause no appreciable downfield shift. At 20% DCA the acid proton resonance in the presence of N methyl acetamide is 0.10 ppm downfield from that in the presence of PBG or in the solvent alone. At high concentrations of acid the chemical shift difference becomes negligible by virtue of equation (6). It is concluded that chemical shift measurements of the acid proton cannot be interpreted quantitatively, are very insensitive to protonation in dilute solutions and do not provide evidence as previously supposed (Stewart et al, 1967) to contradict the concept of charging of peptide groups.

The α CH proton of DCA occurs as a singlet at 4.00 τ. However, this proton resonance can be expected to be upfield when the acid is in the anionic form, $CHCl_2\ COO^-$ (DCA^-). Simple amide studies (this work) have shown that the equilibrium between unprotonated and protonated DCA is rapid and the αCH resonance should move upfield as the proportion of DCA^- in the DCA increases. Under normal

conditions $[DCA] \gg [DCA^-]$ and hence from equation (6) the upfield shift would be very small. Accordingly, measurements of this resonance were made in $CDCl_3$ solutions containing 1-9% of DCA in the presence and absence of PBG ($DP_2 = 21$). The addition of PBG caused a maximum upfield shift of 0.05 ppm in 1% DCA. If one assumes that the total upfield shift from DCA to DCA^- is 0.50 ppm, then a simple calculation shows that there is about 2% charging of the peptide groups of PBG in 1% DCA - 99% $CDCl_3$. This figure is less than that estimated by Hanlon (1966).

PBG IN MIXED SOLVENTS OF DCA AND $CDCl_3$

Previous work in this sytem (Fenn, 1967) had been performed on a Perkin Elmer R10, 60MHz machine. In order to ascertain that the chemical shifts observed for the αCH of PBG were true chemical shifts, some of the original experiments were repeated using a 100MHz machine. The results are illustrated graphically in Figs 14(a), 14(b) and are, in all ways, complimentary to the results obtained at 60MHz. Chemical shifts for the two broad αCH resonances observed are identical with those obtained at 60MHz. Thus the two peaks observed are not in any way due to spin-spin coupling with other protons. As the significance of the presence of the two peaks has been described in great detail elsewhere (Fenn, 1967 and Bradbury and Fenn, 1969, (a), (b)) it is not proposed to discuss it here.

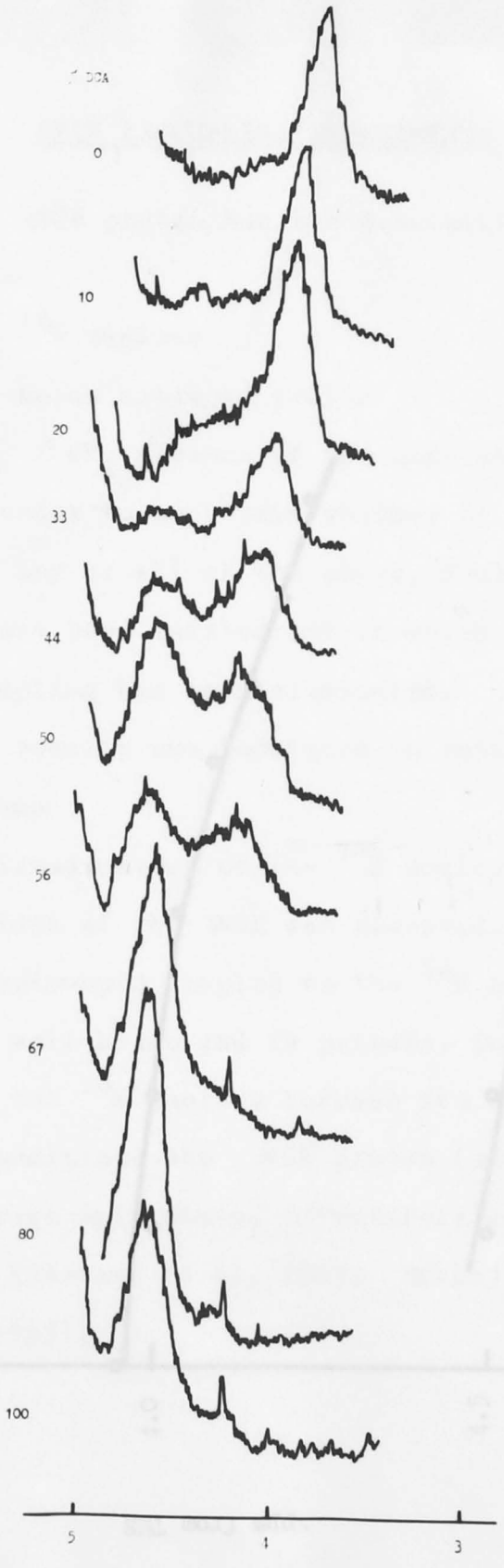


FIG 14(a)

Resonances due to CH of PBG (DP 26)

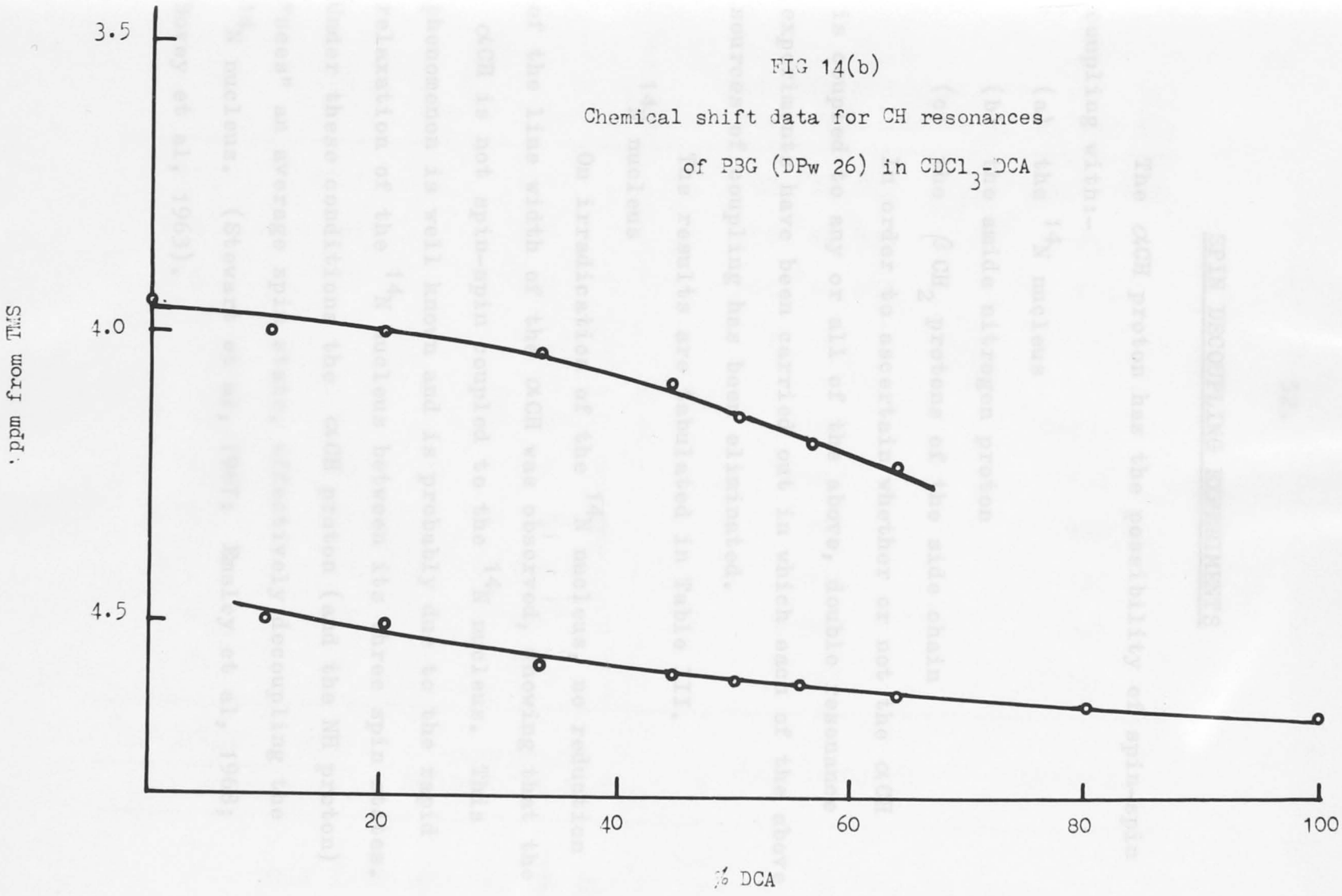
as function of $CDCl_3$-DCA

composition

FIG 14(b)

Chemical shift data for CH resonances

of PBG (DPw 26) in $CDCl_3$-DCA



SPIN DECOUPLING EXPERIMENTS

The αCH proton has the possibility of spin-spin coupling with:-

(a) the ^{14}N nucleus

(b) the amide nitrogen proton

(c) the β CH_2 protons of the side chain

In order to ascertain whether or not the αCH is coupled to any or all of the above, double resonance experiments have been carried out in which each of the above sources of coupling has been eliminated.

The results are tabulated in Table III.

^{14}N nucleus

On irradication of the ^{14}N nucleus, no reduction of the line width of the αCH was observed, showing that the αCH is not spin-spin coupled to the ^{14}N nucleus. This phenomenon is well known and is probably due to the rapid relaxation of the ^{14}N nucleus between its three spin states. Under these conditions the αCH proton (and the NH proton) "sees" an average spin state, effectively decoupling the ^{14}N nucleus. (Stewart et al, 1967; Emsley et al, 1968; Bovey et al, 1963).

TABLE III

SPIN DECOUPLING AT 60MHz OF PROTON RESONANCES OF PBG

SOLVENT	SAMPLE OF PBG[a]	SPIN DECOUPLING EXPERIMENT	LINE WIDTH (Hz)	
			α CH	BENZYL CH_2
$CDCl_3$	PBG	nil	30	10
$CDCl_3$	PBG-d	nil	22	10
TFA	PBG	nil	22	5.0
TFA	PBG	irrad. of ^{14}N	22	5.0
TFA-d	PBG-d	nil	16	5.0
TFA-d	PBG-d	irrad. of B and β CH_2 groups at 7.75	5.3	5.0
TFA	PBG DP_w 295	nil	22	5.0
TFA-d	PBG DP_w 233	nil	16	4.5
DCA-d-$CDCl_3$[b]	PBG DP_w 28	nil	16.5	...

(a) DP_w = 21 except where stated otherwise

(b) Solvent 20% DCA-d-80% $CDCl_3$

TABLE IV

LINE WIDTH (Hz) AT 100MHz OF CH RESONANCES OF PBLG AS FUNCTION OF DP_w AND SOLVENT.

Upfield PBG peak in 95% $CDCl_3$ - 5% TFA; downfield PBG peak in 80% $CDCl_3$ - 20% TFA. One peak visible with very small amounts of the second peak.

DP_w	ACID	UPFIELD PEAK	LINE WIDTH DOWNFIELD PEAK
13	TFA-d	28[a]	19
26	dry TFA	29	28
28	dry TFA-d	15	19
28	TFA-d[b]	17	37
40	TFA-d	20	25

(a) An appreciable amount of the downfield peak present.

(b) Water (3%) in the mixed solvent.

Amide nitrogen proton

On replacement of the amide nitrogen proton by deuterium, there is a marked reduction in the line width of the αCH. Replacement of the NH by ND is achieved by the use of deutero-PBG in $CDCl_3$ or simply by measurements in deutero TFA or deutero DCA.

From Table IV it is seen that sharpening of both the upfield and downfield peaks occurs when deuterated samples are used. Thus there must be some spin-spin coupling of the αCH and the amide nitrogen proton. This coupling also observed by Bovey (1968) indicates a rate of exchange of amide protons with solvent which is relatively slow ($k < 10^{-2}$ sec^{-1}). Similar observations concerning the exchange of this proton were made with simple amides (This work).

Comparison of the line widths of the αCH for PBG-d and PBG in $CDCl_3$ show a small reduction of line width of the αCH on deuteration. Again this is a clear indication of spin-spin coupling of the αCH with the NH. It is also noted that the line width of the αCH is greater in the helical form than in the random coil form. This additional broadening is due to the greater rigidity of the rod-like structure which increases the correlation time of the protons (Emsley et al, 1968).

β CH_2 protons

Decoupling of the β CH_2 protons, achieved by irradiation of the β CH_2 protons in TFA-d, caused the line width of the αCH resonance to fall to a value which is only slightly greater than that of the benzyl CH_2 resonance.

It is apparent that the main contribution to the line width of the αCH resonance is due to coupling with the β CH_2 protons of the side chain. A small contribution can be attributed to coupling with the amide nitrogen proton, but no increase in line width due to coupling with ^{14}N nucleus is observed.

Previous work (Bradbury and Stubbs, 1968; Chapman 1968) had shown that, while the line width of the benzyl and phenyl resonances increases with increase in the DP_w of the rod, the line width was independent of DP_w in the random coil form. From table IV it is seen that the line width of the αCH is similarly independent of DP_w in the random coil form.

From the nmr experimental results presented it is evident that the exchange of the amide nitrogen proton is relatively slow ($\tau > 10^{-2}$ sec). This is true for both the simple amides and the poly-α-amino acid in the solvent systems investigated and is thoroughly consistent with rates observed by other workers (Stewart et al, 1967).

It is also evident that protonation of an amide group by a strong acid causes a downfield shift of the proton resonances adjacent to it and in some cases an increase in the rate of exchange of the amide proton.

On the one hand, interpretation of the spectra of simple amides clearly indicates that the equilibrium between protonated and unprotonated species is generally fast ($\tau < 10^{-2}$ sec). Our interpretation (Bradbury and Fenn, 1969) of the two αCH proton resonances visible in the spectrum of PBG necessitates that this equilibrium be slow. Such data appears to be inconsistent. However, support for the postulate that exchange between protonated and unprotonated species in poly-α-amino acids is slow arises from studies of poly DL amino acids.

Spectra of non helical low DP poly-DL-alanine (Klotz, 1971) and high DP poly-γ-benzyl-DL-glutamate and poly-β-methyl-DL-aspartate (Bradbury et al, 1968 (a)) in

$CDCl_3$ show the same chemical shift for the αCH resonance as does the helical L-polymer in the same solvent.

Previously, we had calculated that there should be a chemical shift difference between poly-DL-alanine and poly-L-alanine of 0.22 ppm in this solvent (Bradbury and Fenn 1969 (a)). However, since our calculations involved extrapolation of experimental data for poly-DL-alanine (Stewart et al, 1967) to zero concentration of acid the discrepancy may be due to this.

Upon addition of acid, the behaviour of the spectrum of poly-DL-alanine is similar to that of poly-L-alanine i.e. a downfield αCH resonance appears which increases in area at the expense of the area of the upfield αCH resonance. It was also observed that small amounts of acid (1%) caused an appreciable downfield shift (0.2 ppm) of the upfield αCH resonance of poly-DL-alanine. Such results imply that the assignment of the two αCH resonances to helical and random conformations is incorrect since both the DL and L polymers show essentially the same chemical shift in $CDCl_3$. Similarly the attribution of low-field and high-field αCH resonances to different

molecular weight fractions of a polydisperse sample of poly-α-amino acid is also not likely since in essence this explanation depends on the assignment of different chemical shifts to helical and random conformation (Ullman, 1970).

The parallel behaviour of DL and L poly alanine is consistent with the assignment of the two αCH resonances (Klotz, 1970) to protonated and unprotonated residues and the postulate (Bradbury and Fenn, 1969 (a)) of slow exchange between the two species.

The reason for the difference in rates between poly-α-amino acids and simple amides may be that the rate of this equilibrium depends on the structure of the amide itself. The results for diethyl acetamido malonate have been interpreted in terms of a slow equilibrium between one isomer, which is in all probability sterically hindered, and the protonated species. As most of the amides examined were simple amides and not sterically hindered, it would be interesting to examine a series of amides which are sterically hindered. Quite possibly the equilibrium between protonated and unprotonated forms in sterically hindered amides (such as a poly-α-amino acid) is slow, the rate determining step being the conversion to a more easily protonated form.

KINETIC SCHEME FOR PBG

In proposing a kinetic scheme for the helix to coil transition, one must satisfactorily account for the following experimental results.

1. At low DP i.e. $\geq$ 100 there are two αCH proton resonances, (Ferretti, 1967; Fenn, 1967; Bradbury and Fenn, 1969 (a); Bradbury et al, 1968; Ferretti and Paolillo, 1969; Klotz, 1971) which indicate that there are two distinct types of αCH protons. Yet at high DP only one αCH resonance is normally observed which increases in line width during the transition (Markley et al, 1967; Fenn 1967, Bradbury et al, 1970).

2. Kinetic studies (Lumry et al, 1964; Hamori and Scheraga, 1967; Hammes and Roberts, 1969; Schwarz and Seelig, 1968) show that the rate of the helix to coil transition in polypeptides of high DP is extremely fast, the relaxation time for PBG being 5×10^{-7} secs at the centre of the transition (Schwarz and Seelig, 1968).

The appearance of two αCH resonances at low DP instead of the single resonance observed at high DP is rather difficult to account for. It has been proposed that this could be due to more rapid exchange between the two αCH species as DP increases (Bradbury E.M. et al, 1968).

This is probably incorrect as recent experimental data has shown the existence of two αCH proton resonances for PBG of high DP. (Ferretti and Ninham 1970). A simpler explanation is that the upfield αCH resonance, like the phenyl and benzyl resonance, broadens with increasing DP (Bradbury and Stubbs, 1968; Chapman, 1968) which causes fusion of the resonances. If this explanation is correct then since the two resonances have different chemical shifts one should observe a broadening of the αCH resonance during the transition. Without exception, such an increase in line width is observed in cases where only one αCH resonance is visible. (Markley et al, 1967; Fenn, 1967; Bradbury et al 1970).

The scheme to be proposed relies on the assignment of the low field resonance to charged residues and the high field resonance to uncharged residues.

The scheme envisages two alternate paths for the conversion of a helical residue h with intramolecular hydrogen bonds intact to a fully protonated random coil residue CH^+

$$h \rightleftharpoons c \qquad \text{fast} \qquad (1)$$

$$c + TFA \rightleftharpoons CH^{+} + TFA^{-} \qquad \text{slow} \qquad (2)$$

$$h + TFA \rightleftharpoons hH^{+} + TFA^{-} \qquad \text{slow} \qquad (3)$$

$$hH^{+} \rightleftharpoons CH^{+} \qquad \text{fast} \qquad (4)$$

Where c represents an uncharged random coil residue involved in hydrogen bonding with the solvent and hH^{+} a helical residue which is also protonated. The latter cannot be easily accommodated in the helix forming highly distorted hydrogen bonds (Hanlon, 1966) and hence the probability of reactions (3) and (4) occurring would be low compared to reactions (1) and (2).

Clearly, the kinetic scheme satisfies the requirement that the transition between helix and coil be rapid. However, the assignment of the two CH resonances necessitates that the equilibrium between charged and uncharged species be slow. Although this equilibrium is rapid in the case of small model amides, apparently it is slow on Klotz's interpretation for poly-α-amino acids as shown by studies on DL poly-α-amino acids (Klotz, 1971).

The double αCH resonance then consists of an upfield peak which is a composite of h and c residues which are in rapid equilibrium and a downfield peak which is composed of hH^{+} and CH^{+} residues in rapid equilibrium. As

stated above the concentration of hH^+ is probably very small.

The increase in area of the downfield resonance at the expense of the upfield resonance is adequately explained by equations (3) and (4). By the law of mass action, increasing the concentration of acid shifts the equilibrium to the right increasing the concentration of CH^+ and hH^+ thus the area of the lowfield resonance and decreasing the concentration of c and h hence the area of the upfield resonance.

The slight downfield shift of both resonances (0.2 ppm for the upfield and 0.1 ppm for the downfield) observed on the addition of acid is more difficult to explain (see Fig. 14). According to previous ideas (Bradbury and Fenn, 1969 (a)) the small downfield shift was attributed to an increasing amount of c and CH^+ in the upfield and downfield resonances respectively. However, since it has been shown that the chemical shifts of unprotonated random and helical residues are identical (Klotz, 1971; Bradbury et al, 1968 (a)) the explanation for the upfield peak shift cannot be correct.

The kinetic scheme envisaged also invalidates the possibility of a rapid equilibrium between the species in this resonance and some protonated species.

It is proposed that the slight downfield chemical shift of the upfield CH resonance is due to an increasing amount of solvation of the residues by acid. That is to say, the acid is hydrogen bonded to unprotonated helical residues. If this explanation is correct, then the increase in hydrogen bonding of the acid should be reflected in a downfield shift of the acid proton resonance with increasing acid content of the solvent. Such an effect has been noted by several authors (Stewart et al, 1967; Liu and Lignowski, 1970). The observation that the addition of a small amount of acid (1%) to a $CDCl_3$ solution of poly-DL-alanine causes an immediate downfield shift of 0.2 ppm of the upfield resonance supports this view (Klotz, 1971). Additional acid causes no further shift of this resonance. One would expect that the random poly-DL-alanine would be less sterically hindered, hence more easily solvated, than the helical poly-L-alanine. Thus the initial downfield shift observed.

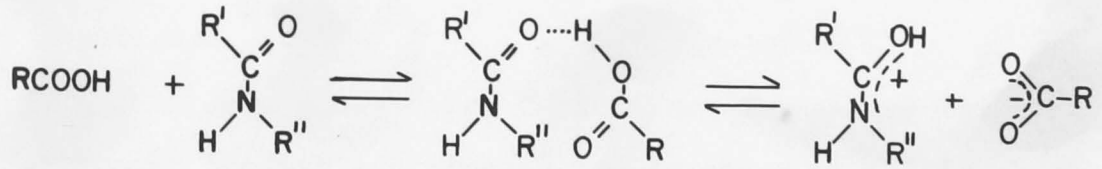
If increasing acid content of the solvent causes increasing solvation then the ratio of solvated residues to unsolvated residues should increase in the upfield resonance. Hence the rate at which the upfield resonance shifts should increase with increasing acid content of the solvent. This change of rate is observed experimentally (Fig 14).

The above does not in any way invalidate the kinetic scheme proposed but merely indicates the complex nature of the transition. Hence the original assignments (Bradbury and Fenn, 1969 (a)) are modified to include solvated C and H residues in the upfield resonance.

MECHANISM OF THE HELIX TO COIL TRANSITION

In attempting to postulate a mechanism by which the helix to coil transition occurs, one must take into account the following: the strong binding of acid to the side chain carboxyls (Milstein and Charney, 1970; this work), the protonation observed at high concentrations of acid (Fenn, 1967; Bradbury and Fenn, 1969 (a)) and the undoubted solvation of the poly-α-amino acid by the acid during the transition (Liu et al, 1970).

If one assumes that protonation is an extreme case of hydrogen bonding, then strong hydrogen bonding must be considered as precursory to protonation. Thus there must be at some stage during protonation a hydrogen bonded intermediate such as illustrated below.



By the law of mass action, additional acid pushes the equilibrium to the right and protonation ensues.

On the basis of the above, protonation of the amide groups of polypeptides must be preceded by strong hydrogen bonding of the acid to the amide groups.

The mechanism can be described as follows. On the initial addition of acid, strong binding of the acid to the side chain carboxyls occurs. Increasing the acid concentration increases the activity of the acid and hydrogen bonding to the backbone takes place. A further increase in acid concentration causes protonation and consequent disruption of the helix.

If the equilibrium between the strongly hydrogen bonded species and the protonated species is drastically disturbed by only a small additional amount of acid then one may present a possible explanation for the apparent conflict of opinion over the two possible mechanisms postulated.

The above being correct one may expect both hydrogen bonding and protonation to occur at the transition point. Thus both will be detected. However, which mechanism causes the actual collapse of the helix is still a matter for conjecture.

SECTION II:

A NEW METHOD FOR DETERMINING THE SEQUENCE OF AMINO ACIDS IN PEPTIDES

INTRODUCTION

Technologically, one of the most difficult problems associated with protein chemistry is the determination of the primary structure or sequence of amino acid residues in proteins. Briefly the general procedure can be described as follows:

(a) fragmentation of the protein into smaller peptide fragments.

(b) separation and purification of fragments.

(c) systematic end group analysis and stepwise degradation.

(d) correlation of peptide fragments from overlapping sequences.

(a) Fragmentation of Proteins

In the choice of a suitable method of degradation one must take into account that the reagent should cause a minimum of side reactions, exhibit a desired specificity and not cause rearrangement of peptide bonds in the protein. Although partial acid hydrolysis is the oldest (Fischer and Alberhalden, 1906) and the most commonly used method of fragmentation, the method suffers from the disadvantages of destroying tryptophan, glutamine and asparagine and, moreover, causes rearrangement of serine and threonine residues where the acyl group migrates from nitrogen to oxygen (Desnuelle and Casal, 1948; Elliott, 1952).

Generally, fragmentation by partial acid hydrolysis is conducted in conjunction with some other method such as enzymic hydrolysis. Hydrolysis by enzymes is a milder method of fragmentation but again since most enzymes are non-specific a complex mixture of peptides results. More specific points of fragmentation are obtained through chemical cleavage by such reagents as N-Bromosuccinimide (Han et al, 1966; Ramachandran and Witkop, 1967). An excellent review on the chemical cleavage of proteins has recently become available (Spande et al, 1970).

Although fragmentation of the protein can be achieved by a variety of methods, the main problem is to ascertain the conditions necessary to produce the minimum degree of complexity in the resulting mixture of peptides. An increase in complexity of mixtures of closely related compounds requires an increase in sensitivity of methods of fractionation, thus it is necessary to limit the complexity as much as possible.

(b) Separation of fragments

With the great variety of methods available, paper, ion exchange and column chromatography, electrophoresis counter current distribution, dialysis and gel filtration,

highly complex mixtures of peptides can be fractionated.

As the technique of separation of protein hydrolyzates is not within the scope of this thesis it is not proposed to discuss fully separation of fragments. Excellent reviews on the subject are available (Canfield and Anfinsen, 1963; Leggett Bailey, 1967).

(c) Determination of Sequence

The peptide chain in its general form may be represented as follows:

NH_2-CHR^1-CO-NH-CHR^2-CO-NH ..... CO-NH-CHR^n-CO_2H

In accordance with the suggestion of Sanger (1952), the amino acid residue bearing the free αamino group or in the case of proline, imino, is called the N terminal whereas that residue at the end of the chain bearing the free αcarboxyl is referred to as C-terminal.

There are several criteria for any method of sequenation which are highly desirable.

The sequenating step must be quantitative. Non-quantitative reactions limit the extent to which satisfactory results can be obtained along the chain, a stage being reached where large numbers of different amino acid residues are released at each step, the different amino acid residues originating from chains where all amino acid residues have been incompletely removed. Such a mixture of

residues makes correct determination of the amino acid residue in the sequence impossible.

In view of the fact that many proteins contain a large number of amino acid residues, it is desirable that the reaction be fast and not involve many steps. Solvents used in the process should be easily obtainable and easily purified to a high degree. An important consideration is that the peptide or protein be soluble in the reaction media. As the molecular weights of proteins are very large and in most cases only a small amount is available for sequenation the method used for characterising amino acid derivatives should be highly sensitive. In the past many elegant and effective methods have been devised for the sequenation of proteins and it is proposed to present a short summary of these methods.

1. Mass Spectrometry

Mass spectrometry shows great promise as a method for elucidation of the sequenation of small peptides. Small quantities (10-100 μg) are required, the method is rapid and there are possibilities of automation involving the use of computers (Senn and McLafferty, 1966). The

outstanding problem which has occurred was the discovery of peptide derivatives which are sufficiently volatile to be used in the mass spectrometer. Biemann (1959, 1960) achieved volatility by reduction to the peptide alcohol with $LiAlH_4$. Later authors used N acylated derivatives, principally the N-trifluoroacetyl, of methyl, ethyl and tertiary butyl esters (Weygand et al, 1956, 1963), fairly satisfactory sequenation being achieved up to five residues. Observations that while many peptidolipids were volatile enough to be used in the mass spectrometer peptides of equal chain length were not, indicated that the important factor controlling the volatility of peptide derivatives was the presence or absence of tertiary amide groups (Lederer, 1968). Apparently, the presence of amide protons in facilitating the formation of secondary structure lowers the volatility. Such increase in volatility on the loss of amide protons was aptly demonstrated by Das et al (1969) and de Haas et al (1969) who found that while a heptapeptide derivative was not volatile enough to give a mass spectrum, the completely N-methylated peptide gave a satisfactory spectrum.

However, mass spectra may be complicated by the presence of certain amino acids in the peptide such as, lysine, arginine, methionine, glutamic and aspartic acids.

The problem appears to be not only loss of volatility due to the ionic groups but the appearance of unusual splitting patterns which complicate the spectrum (Lederer, 1968). Possibly, preparation of suitable derivatives could eliminate this problem.

Even so, permethylation has enabled Agarwal et al (1969) to determine unequivocally the sequence of a heptadecapeptide using 180 n. moles of material, hepta and octapeptides being commonly sequenated by the method (Lederer, 1968).

While the method shows great promise, there are several problems concerning the abnormal amino acids to be solved, and, of course, isomers of amino acids cannot be distinguished.

Finally, mention should be made of a more conventional use of mass spectrometry for sequenation. This is the use of a volatile Edman reagent and the sequence determined by the normal Edman technique, the amino acid residue being characterised by mass spectrometry (Richards et al, 1969).

2. Nuclear magnetic resonance spectroscopy

The great advantage of nmr spectroscopy over existing methods is that it is wholly non-destructive. However, there are major disadvantages associated with use of the method even for peptides of moderate size.

The nmr spectrum of a peptide should approximate the sum of the spectra of its constituent amino acids (Jardetzky and Jardetzky, 1957) with differences being due to inclusion of the amino acid in the primary chain and effects due to secondary and tertiary structure if present. By using the effect of primary structure on the glycine CH_2 on inclusion into a peptide chain, Nakamura and Jardetzky, (1967, 1968) have been successful in assigning resonances unequivocally to particular residues in a series of dipeptides and oligopeptides. However, with the exception of glycine, the nature of an amino acid residue can be determined from the spectrum only by its side chain resonances not by its αCH resonance. Since the side chain resonances are sensitive to the titration of the terminal amino and carboxyl groups but not to other aspects of primary structure, only the N-terminal and C-terminal residues can be determined directly. Di and tripeptides have been sequenced in this way (Sheinblatt, 1966).

In theory, it should be possible to extend the method to longer peptides by using the nearest neighbour titration shift of the αCH. However, the difficulty in not only resolving the various αCH resonances for peptides larger than two of three residues (Fenn, 1967) but associating the αCH resonance with the correct amino acid residue increases very rapidly with increase in chain length. Furthermore, shifts due to close proximity of other residues further removed in the primary sequence, brought about by folding of the primary chain, would add more confusion to the correct assignment of αCH resonances.

It can be concluded that sequenation by nmr spectroscopy is likely to be restricted to very short peptides.

3. Enzymic degradation

The enzymes, leucine aminopeptidase and carboxypeptidase A and B are specific for N-terminal and C terminal residues respectively. Enzymic hydrolysis is limited in that digestion does not discontinue after removal of the first residue, the new terminal residue being subjected to attack by the enzyme resulting in a complex mixture of amino acids. However, since the relative rates of hydrolysis of different amino acids are often dissimilar (Smith and

Hill, 1960) it is possible to obtain partial elucidation of the sequence from kinetic studies, up to six residues in the case of leucine amino peptidase (Bailey, 1967) and three for carboxypeptidase (Harris and Li, 1955). Such techniques become difficult when adjacent terminal residues are similar.

4. Chemical methods

(i) C-terminal degradation

At the present state of the science no fully satisfactory chemical technique exists for sequential degradation of peptides from the carboxyl terminus ~~exists~~. Mention must be made of two methods which have to a small extent provided some information about the C terminal sequence. Stark (1968) using ammonium thiocyanate and acetic anhydride sequentially degraded a number of peptides with interpretable results, typically for two or three stages, but in the best case for six stages. The method is limited in that carboxyl terminal aspartic and proline residues are not removed and the reaction is not quantitative. N peptidyl alcohols have received some attention as possible routes to sequenation. Such alcohols are readily available from the peptide ester by reduction with a metal hydride (Leggett Bailey, 1955 (a)). Acid reagents induce an N,O acyl migration to form the β amino ester which on reduction liberates the C-terminal amino

alcohol (Leggett Bailey, 1955 (b)). The method again suffers from the disadvantage of being non quantitative.

(ii) N-terminal degradation

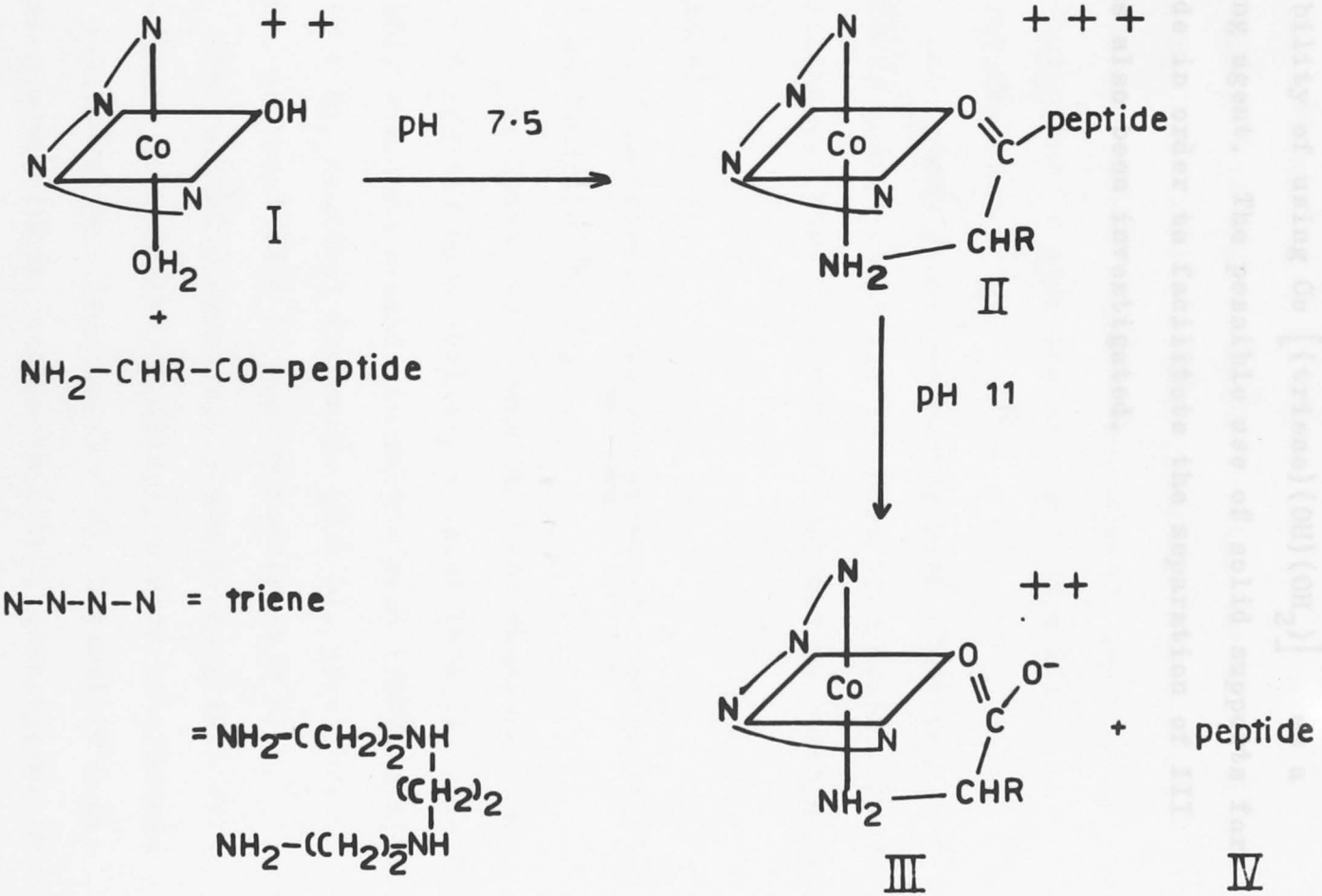
With very few exceptions, knowledge of peptide sequence rests almost entirely on the results obtained by stepwise degradation with phenyl isothiocyanate, the Edman reagent (Edman, 1950). Phenyl isothiocyanate is reacted with the free αamino group in a mildly alkaline buffer and the phenyl thiocarbamyl peptide so produced separated from reagents and solvents. The terminal residue is then cleaved off as a thiazolinone in anhydrous acid and is converted to the more stable phenyl thiohydantoin in aqueous acid after separation from the peptide and the phenylthiohydantoin characterised by chromatography. Subtle variations of the above basic procedure include the use of volatile Edman reagents and the characterisation of the resulting phenyl thiohydantoin by mass spectrometry (Richards et al, 1969) and the combination of 'Dansyl' end group determination and Edman degradation (Gray and Hartley, 1963). Other variations include the use of a solid support for the peptide. When the peptide is attached to a solid support, the mechanics of performing degradations becomes greatly simplified since components are easily separated and extractions can be avoided. An excellent review of developments in this

technique by Stark (1970) is available. All solid state degradations known to date employ the Edman technique.

Although the Edman technique is the most widely used method, it is not without difficulties. Among them are the solubility problems associated with small peptides which leads to a rapid drop in yield as a degradation approaches the C-terminal. Furthermore, it has been shown (Kopple and Bachli, 1959) that the rigorous conditions used to cleave the thiazolinone, anhydrous trifluoroacetic acid, may cause protolysis of internal peptide bonds. Thus non-quantitative conditions are brought about. It is quite obvious that milder methods using a more versatile solvent would be desirable.

Such a method suggests itself from work carried out by Buckingham et al (1967). Buckingham et al observed that the cobalt complex Co $\left[(\text{triene})(OH)(OH_2)\right]^{++}$ (I) could be reacted with a C-terminal protected amino acid or peptide in aqueous solutions and pH 7.5-8.0 to form an amino acid or peptide complex (II) in good yield. The peptide complexes were found to be stable compounds and could be isolated. Raising the pH to 10.5-11 of a solution of the peptide complex hydrolysed the now highly activated peptide bond giving a highly coloured amino acid complex (III) and the peptide less one residue (IV) (See Fig 15).

Fig 15


pH 7·5
peptide
CHR
pH 11
N-N-N-N = triene
= NH2-(CH2)2-NH
(CH2)2
NH2-(CH2)2-NH
+ peptide

It is the aim of this thesis to investigate the possibility of using Co $\left[(\text{triene})(OH)(OH_2)\right]^{++}$ as a sequenating agent. The possible use of solid supports for the peptide in order to facilitate the separation of III and IV has also been investigated.

MATERIALS

Pyridine acetate

Equimolar quantities of freshly distilled acetic acid (M & B, B.P. 114-115°C) and pyridine (M & B, B.P. 112-114°C) were mixed, the reaction mixture cooled and dissolved in water to give the required molarity.

Tetrahydrofuran

Laboratory grade tetrahydrofuran (BDH) was refluxed over sodium metal for 12 hours then distilled. The distillate was then redistilled over sodium borohydride B.P. 65°C.

Hydrochloric acid

6N hydrochloric acid was prepared from constant B.P. acid as described by Vogel.

The following were reagent grade chemicals and used without further purification, ethanol (M & B), triethylamine (BDH), hydrogen bromide in acetic acid (BDH), ethylene diamine (M & B), dimethyl formamide (M & B), perchloric acid (BDH), methanol (M & B) sodium iodide (M & B).

The following chemicals (laboratory grade) were used: carbonyl diimidazole (Aldrich), sodium bicarbonate (M & B), p-amino methyl toluene (M & B), butanol (M & B), sodium perchlorate (BDH), ninhydrin (BDH), isopropanol (M & B),

ammonium bicarbonate (M & B), carbobenzoxy chloride (Fluka), DL leucyl-glycyl-DL phenylalanine (NBC), glycyl DL phenylalanine (NBC), glycyl-DL tyrosine (NBC), glycyl-DL leucine (NBC), DL alanine (BDH), glycine (BDH), L arginine (NBC), L aspartic acid (NBC), cysteic acid (BDH), L glutamic acid (NBC), histidine (BDH), isoleucine (NBC), DL leucine (NBC), L lysine (NBC), L phenylalanine (NBC), L proline (NBC), L serine (NBC), L tryptophan (NBC), L tyrosine (NBC), DL valine (NBC), DL norleucine (NBC).

$Co[(triene)CO_3]\,Cl.H_2O$ was obtained from Dr. D.A. Buckingham, Research School of Chemistry, A.N.U.

Resins

The following resins and ion exchange media were employed. (Table V).

Resin	Supplier	Type
Merrifield	Sigma	chloro methyl polystyrene
IR45	Amberlite	Weakly basic ion exchange
CG45	Amberlite	Weakly basic ion exchange
AG 3	BioRad	Weakly basic ion exchange
1 x 10	Dowex	Strongly acidic ion exchange
50 x 2	Dowex	Strongly acidic ion exchange
CM Sephadex	Pharmacia	Weakly acidic ion exchange
Amino ethyl cellulose	Whatman	Weakly basic ion exchange

EXPERIMENTAL

1. Carbobenzoxy-DL-alanine

17.8 grams (0.2 moles) of DL-alanine and 42 grams of sodium bicarbonate were suspended in 250 mls of water in a litre beaker and the mixture stirred vigorously. 30 mls of carbobenzoxy chloride was added to the mixture in five equal amounts over a period of 30 minutes. The stirring was continued for two hours longer and the reaction mixture then extracted once with 20 mls of ether. The aqueous fraction was carefully acidified to pH2 by dropwise addition of 6N HCl. After storing at $0^{o}C$ for 2 hours the crystals of carbobenzoxy alanine which separated were filtered off, dried at $50^{o}C$ and recrystalised from ethyl acetate/petroleum ether. Yield 35 grams (80%) M.P. 112-113^{o}C.

2. Carbobenzoxy-glycine

15 grams (0.2 moles) of glycine was converted to carbobenzoxy-glycine by a similar procedure. The crude product was recrystalised from chloroform.
Yield 34.5 grams (82%) M.P. 118-119^{o}C.

3. Carbobenzoxy-DL leucyl-glycyl-DL phenylalanine

1 gram (0.003 moles) of DL leucyl-glycyl-DL phenylalanine and 1 gram of sodium bicarbonate were suspended in 10 mls of water in a 30 ml beaker and the mixture stirred vigorously. 1 gram of carbobenzoxy chloride was added in five equal portions over a period of 30 minutes. The

stirring was continued for 2 hours longer and the reaction mixture then extracted once with 5 mls of ether. The aqueous layer was carefully acidified with 6N HCl to pH2 and the resultant oil which separated extracted into 15 mls of ethyl acetate. The ethyl acetate layer was washed twice with 5 mls of water, dried over anhydrous sodium sulphate. Addition of n-hexane precipitated the crude carbobenzoxy-leucyl-glycyl-phenylalanine. The product was recrystalised from ethyl acetate/n-hexane. Yield 0.9 grams (70%). The product was characterised by nmr spectra.

4. Carbobenzoxy-DL-alanyl-DL-leucine ethyl ester

22.3 grams of carbobenzoxy-DL alanine (0.1 moles) were dissolved in 50 mls of dry, freshly distilled tetrahydrofuran and 16.2 grams of carbonyl diimidazole (0.1 moles) added. After stirring for one hour at room temperature in a vessel protected from atmospheric moisture by a silica gel guard tube, 19.6 grams of DL leucine ethyl (0.1 moles) ester were added. The mixture was stirred overnight at room temperature. After this time, 50 mls of 1N HCl were added and the yellow oil which separated extracted into ethyl acetate. The ethyl acetate layer was washed three times with 30 mls of 1N sodium bicarbonate then once with 30 mls of water. After drying over anhydrous calcium sulphate, the crude product was precipitated by the

addition of n-hexane. Recrystalisation was effected from ethyl acetate and h-hexane.

Yield 33 grams (91%). The product was characterised by nmr spectra.

5. Carbobenzoxy-DL alanyl-DL leucine

33 grams (0.91 moles) of carbobenzoxy-DL alanyl-DL leucyl ethyl ester were dissolved in 100 mls of ethanol and four grams (0.1 mole) of NaOH dissolved in 15 mls of water added. After stirring for one hour, 150 mls of water were added and the stirring continued for another 2 hours. The aqueous solution was acidified to pH2 with 1N HCl and the resulting oil which separated extracted into ethyl acetate. The ethyl acetate layer was extracted five times with 30 mls of 5N sodium bicarbonate, the bicarbonate extractions were combined and acidified to pH2 with 6N HCl. The carbobenzoxy-DL alanyl-DL leucine separated as oil which was extracted into ethyl acetate. Removal of the ethyl acetate under high vacuum at room temperature resulted in a non recrystalisable oil. The product was characterised by nmr spectra.

6. Carbobenzoxy-DL alanyl-DL-leucyl-glycine ethyl ester

16.75 grams (0.05 moles) of the oil, carbobenzoxy-DL alanyl-DL leucine were dissolved in 50 mls of dry,freshly distilled tetrahydrofuran and 8.1 grams (0.05 moles) of carbonyl diimidazole added. After stirring for one hour in

a vessel protected from atmospheric moisture, 7 grams of glycine ethyl ester were added. The mixture was stirred overnight, after which time 50 mls of ^{N} HCl were added and the aqueous solution extracted three times with 50 mls of ethyl acetate. The ethyl acetate extracts were combined and washed three times with 30 mls of 1N sodium bicarbonate and once with 30 mls of water. After drying over anhydrous calcium sulphate, the crude product was precipitated by the addition of n-hexane. Recrystallisation was effected from ethyl acetate/n-hexane.

Yield 17.5 grams (83%). The product was characterised by nmr spectra.

7. Carbobenzoxy-DL alanyl-DL leucyl-glycinol

Method 1

4.2 grams (0.01 moles) of carbobenzoxy-DL alanyl-DL leucyl-glycine ethyl ester were dried at 70°C under high vacuum for three hours. 220 mg (0.01 moles) of $LiBH_4$, dissolved in 5 mls of dry, freshly distilled tetrahydrofuran was added to the peptide and the solution allowed to stand in a sealed vessel for 18 hours at room temperature. After this time 10 mls of N HCl were added and after 1 hour the resultant non recrystalisable oil which separated extracted into ethyl acetate.

Method 2

1 gram (2.5 moles) of carbobenzoxy-DL-alanyl-DL leucine ethyl ester was hydrolysed to carbobenzoxy-DL-alanyl DL leucine by the method previously described (See 5). The resultant oil was dried under high vacuum at room temperature for two hours, dissolved in 10 mls of dry, freshly distilled tetrahydrofuran and 0.41 grams of carbonyl diimidazole added, the stoppered solution being allowed to stand at room temperature for one hour. After this time, 0.25 grams of ethanolanine were added. The solution was stored overnight, then 10 mls of 1N HCl added. The resultant non crystalisable oil which separated was extracted into ethyl acetate (10 mls). The ethyl acetate fraction was washed three times with 10 mls of 1N sodium bicarbonate and twice with 10 mls of water, then dried over anhydrous calcium sulphate.

8. DL alanyl-DL leucyl-glycinol

(a) The resultant oil from method 1 above was transferred to a 250 mls conical flask containing 100 mls of ethyl alcohol and 0.5 grams of palladium oxide catalyst. The palladium oxide catalyst was prepared from palladium chloride according to the method of Greenstein and Winitz (1961). After passing hydrogen over the vigorously stirred solution for six hours the catalyst was filtered off and the ethanol solution evaporated to dryness under high vacuum at room temperature, yielding a white, highly hygroscopic solid. Yield 2 grams (82%). The product was

characterised by nmr spectra.

(b) The resultant oil from method 2 above was reduced to DL alanyl-DL leucyl-glycinol as described in (a) above. The product was again highly hygroscopic. Yield 0.23 grams (35%). The product was characterised by nmr spectra.

(c) 1.1 grams (3m moles) of DL alanyl-DL leucyl-glycine methyl ester hydrochloride was dried under high vacuum at 70°C for three hours. 0.2 grams of $LiBH_4$, dissolved in 5 mls of tetrahydrofuran added and the solution allowed to stand for 18 hours at room temperature. After this time, 10 mls of N HCl added and after one hour at room temperature, the pH raised to 7 with 1M NaOH. The neutral solution was evaporated to dryness under vacuo (40-50°C) and dissolved in a small amount (4-5 mls) of water. The solution was applied to a short column (1 x 8 cms) of Dowex 50 x 8 (Hydrogen form) and the peptide alcohol eluted with 0.1 ammonium hydroxide solution. The peptide alcohol travels down the column as a visible band adjacent to the neutralisation front. The ammonium hydroxide solution containing the peptide alcohol was evaporated to dryness under vacuo yielding a white highly hygroscopic solid.

Yield = 0.5 grams (78%). The product was characterised by nmr spectra..

The nmr spectra of all three samples of DL alanyl-DL leucyl-glycinol were identical.

9. Carbobenzoxy-DL alanyl-glycinol

2.23 grams (0.01 moles) of carbobenzoxy-DL alanine was dissolved in 10 mls of freshly distilled tetrahydrofuran and 1.62 grams (0.01 moles) of carbonyl diimidazole added. The stoppered solution was allowed to stand for one hour at room temperature after which time 0.61 grams of ethanolamine was added. The stoppered solution was stored overnight then 10 mls of 1N HCl added. The yellow oil which separated out was extracted into ethyl acetate. The ethyl acetate layer was washed three times with 10 mls of 1N sodium bicarbonate, twice with 10 mls of water and dried over anhydrous calcium sulphate. Addition of petroleum ether precipitated the crude carbobenzoxy-DL alanyl-glycinol. Recrystalisation was effected from toleune/petroleum ether.

Yield 1.6 grams (57%) M.P. 101-105°C.

10. DL alanyl-glycinol

1 gram of carbobenzoxy-DL alanyl-glycinol was reduced to DL alanyl-glycinol with hydrogen and a palladium catalyst as previously described. The ethanolic solution of DL alanyl-glycinol was evaporated to dryness under vacuo and the residue dissolved in 10 mls of water. The unreacted carbobenzoxy-DL alanyl-glycinol was filtered off and the aqueous solution evaporated to dryness under high vacuo to yield a white, highly hygroscopic solid. Yield 0.5 grams

(35%). The product was characterised by nmr spectra.

11. DL alanine ethyl ester hydrochloride

DL-alanine (5 grams) was suspended in 100 mls of ethyl alcohol and 7 mls of freshly distilled thionyl chloride added dropwise with shaking. After all the thionyl chloride had been added the mixture was refluxed one hour, cooled and evaporated to a thick oil under vacuo at a low temperature. The crude ester was precipitated by the addition of ether. Recrystallisation was effected from the mother alcohol and ether.

Yield 6.9 grams (80%) M.P. 86-87°C. The product was characterised by nmr spectra.

Yields and M.P.t's of the various esters prepared by a similar procedure to the above are given in Table VI.

TABLE VI

Amino acid	Ester	Yield %	M.P.
DL alanine	Ethyl	83	86-87
L arginine	Methyl	97	192-3
L aspartic acid	dimethyl	89	114-5
L histidine	methyl	83	200-1
DL leucine	ethyl	92	119-120
L lysine	ethyl	90	144-5
DL norleucine	ethyl	83	75-76
L phenylalanine	ethyl	88	158-9
L serine	methyl	88	160-1
L Tyrosine	methyl	95	186-7
DL Valine	ethyl	73	101-3
L Cysteic acid	methyl	90	222-3
DL isoleucine	ethyl*		

* isolated only as a non crystalisable oil

12. Glycyl-glycine methyl ester hydrochloride

Glycyl-glycine (2 grams) was suspended in 50 mls of methyl alcohol and 5 mls of freshly distilled thionyl chloride added dropwise with cooling. The mixture was refluxed for two hours, cooled and undissolved material filtered off. The filtrate was concentrated to an oil under

vacuo and the product precipitated by the addition of ether. Recrystallisation was effected with the mother alcohol and ether.

Yield 2.1 grams (84%) M.P. 230°C (decomposes).

13. Glycyl-glycine isopropyl ester hydrochloride

Glycyl-glycine (2 grams) was suspended in 50 mls of isopropyl alcohol and 5 mls of freshly distilled thionyl chloride added dropwise with cooling. The mixture was refluxed for two hours. The ester separated out on cooling. 10 mls of ether were added and the crude product filtered off. Recrystallisation was effected from methanol/ether.

Yield 2.7 grams (85%) M.P. 215-17.

14. Glycyl-DL phenylalanine isopropyl ester hydrochloride

Glycyl-phenylalanine (700 mg) was converted to the isopropyl ester hydrochloride by a method similar to that used for the preparation of glycyl-glycine isopropyl ester hydrochloride.

Yield 800 mg (89%). The product was characterised by nmr spectra.

15. DL leucyl-glycyl-DL phenylalanine methyl ester hydrochloride

DL leucyl-glycyl-DL phenylalanine (1 gram) was converted to the methyl ester hydrochloride by a method similar to that used for glycyl-glycine methyl ester.

Yield 1.0 grams (83%). The product was characterised by nmr spectra.

16. DL alanyl-DL leucyl-glycine methyl ester

DL alanyl-DL leucyl-glycine (1 gram) was dissolved in 10 mls of methyl alcohol, 0.3N in ~~the~~ HCl and allowed to stand in a stoppered vessel for 12 hours. The crude ester was precipitated by the addition of ether and recrystallised from methanol/ether.

Yield 1.1 grams (85%). The product was characterised by nmr spectra.

17. DL leucyl-glycyl-DL phenylalanine isopropyl ester

DL leucyl-glycyl-DL phenylalanine (1 gram) was converted to the isopropyl ester hydrochloride by a method similar to that used to prepare glycyl-glycine isopropyl ester hydrochloride.

Yield 1.0 grams (83%). The product was characterised by nmr spectra.

18. Glycyl-glycine isopropyl ester hydroperchlorate

Glycyl-glycine isopropyl ester hydrochloride (2 grams) was dissolved in methyl alcohol (10 mls) and solid lithium perchlorate (1 gram) added. On cooling the glycyl-glycine isopropyl ester hydroperchlorate which separated was filtered off. Recrystallisation was from methanol ether.

Yield 1 gram (50%).

19. Glycyl-glycine tertiary butyl ester hydrochloride

Glycyl-glycine (1 gram) was dissolved in 10 mls of water and an equimolar amount of silver nitrate (1.29 grams) added. The resulting solution was evaporated to dryness in vacuo at room temperature. The residue was powdered and suspended in 50 mls of ether to which 5 mls of tertiary butyl chloride had been added. After shaking overnight, the insoluble material in the mixture was filtered off and the solution evaporated to dryness. The resultant oil was dissolved in ether and glycyl-glycine tertiary butyl ester hydrochloride precipitated by bubbling hydrogen chloride gas through the solution.

Estimated yield 5%. The product was characterised by nmr spectra.

20. L Phenylalanine tertiary butyl ester hydrochloride

L Phenylalanine (5 grams) was suspended in 100 mls of dioxane to which 2.5 mls of concentrated sulphuric acid had been added and isobutylene passed through the solution for four hours. The reaction vessel was then sealed and allowed to stand at room temperature for four days. After this time 100 mls of water and 10 grams of solid sodium carbonate were added. The apparent pH was increased to 11 by the addition of 2N NaOH and the aqueous layer extracted six times with 50 mls of ether. The ether extracts were combined, concentrated to a

small volume (10 mls) under vacuo and hydrogen chloride bubbled through the solution. The precipitated phenylalanine tertiary butyl ester hydrochloride was filtered off and re-crystallised from ethyl acetate/n-hexane.

Yield 3.4 grams (40%) decomposes on heating.

21. Glycyl-DL tyrosine tertiary butyl ester, glycyl-DL leucine tertiary butyl ester

The tertiary butyl esters of glycyl-DL tyrosine and glycyl-DL leucine could not be prepared by either of the two above methods.

22. DL-alanine polystyrenate

1 gram of Merrifield resin i.e. poly (chloromethyl styrene), (1.3 meq chlorine) was suspended in 15 mls of absolute alcohol, 300 mg (1.3 m moles) of carbobenzoxy-DL alanine and 130 mg (1.3 m moles) of triethylamine added, and the mixture refluxed for 60 hours. After this time, the resin was filtered off, washed three times each with 10 mls of ethanol, 1M sodium bicarbonate, water and ethanol, then dried under high vacuum at 70°C. The dried resin was suspended in 10 mls of hydrogen bromide in acetic acid in a sealed vessel. After stirring the mixture for one hour, the resin was filtered off, washed three times each with 10 mls of glacial acetic acid, water and ethanol, then dried under high vacuum at 70°C.

6.57 mg of the substituted resin was heated with 3 mls of 6N HCl at 110°C. After 24 hours the resin was filtered off and washed with 10 mls of 0.1M HCl. The filtrate and washings were combined, evaporated to dryness and the residue dissolved in 10 mls of 0.1M HCl. 0.5 mls of this solution was analysed on a Technicon Amino Acid Analyser according the method of Bradbury et al (1965) for a value of 0.04 μM of alanine. This is equivalent to 120 μM of alanine per gram of Merrifield resin.

23. DL leucyl-glycyl-DL phenylalanine poly styrenate

DL leucyl-glycyl-DL phenylalanine was coupled to one gram of Merrifield resin by a similar procedure.

Amino acid analysis of a sample of the resin (8 mg) gave the following:-

leucine 50 μM per gram

glycine 52 μM per gram

phenylalanine 53 μM per gram

24. poly (2 amino ethyl) amino methyl styrene

Merrifield resin (5 grams) was heated to 120°C with 20 mls of ethylene diamine for 10 minutes. After this time, the modified resin was filtered off, washed three times each with 10 mls of water and ethanol, then dried under high vacuum at 70°C.

25. poly (2- DL alanyl ethyl) amino methyl styrene

300 mg of carbobenzoxy-DL alanine (1.3 m moles) was dissolved in 10 mls of dry, freshly distilled tetrahydrofuran and 1.5 grams (6.5 m moles) of carbonyl diimidazole added. After one hour at room temperature in a sealed vessel, 1 gram of poly (2 amino ethyl) amino methyl styrene was added and the mixture stirred for eight hours. After this time, the resin was filtered off, washed thrice each with 10 mls of ethanol, 1N sodium bicarbonate, water and ethanol, then dried under high vacuum at 70°C.

The carbobenzoxy group was removed as described above. Amino acid analysis of a sample of the resin (50 mg) revealed 70 M of alanine per gram of resin.

26. poly (2-DL leucyl-glycyl-DL phenylalanyl ethyl) amino ethyl styrene

DL leucyl-glycyl-DL phenylalanine was coupled to one gram of the modified resin by the procedure outlined above. Amino acid analysis of a sample of the resin (25 mg) gave the following:-

leucine	40 μM per gram
glycine	39 μM per gram
phenylalanine	40 μM per gram

27. DL leucyl-glycyl-DL phenylalanyl ethyl cellulose

Carbobenzoxy DL leucyl-glycyl-DL phenylalanine was coupled to amino ethyl cellulose by the procedure described for poly (2-alanyl ethyl) amino ethyl styrene . However, on the addition of hydrogen bromide in acetic acid, the substituted cellulose dissolved. An estimate of the amount of peptide coupled was obtained in the following way.

1 gram of substituted cellulose was dissolved in 10 mls of hydrogen bromide in acetic acid. After one hour the solution was evaporated to dryness under vacuo and the residue incubated with 3 mls of 6N HCl at 110°C in a sealed tube for 24 hours. After this time the solution was evaporated to dryness and the residue dissolved in 10 mls of 0.1 N HCl. Amino acid analysis of this solution gave the following:-

leucine	63 μM	per gram of amino ethyl cellulose
glycine	65	"
alanine	66	"

28. Coupling of DL leucyl-glycyl-DL phenylalanine to weakly basic ion exchange resin IR45

250 mg of carbobenzoxy-DL leucyl-glycyl-DL phenylalanine was dissolved in 10 mls of dry dimethyl formamide and 1.5 grams (6.5 m moles) of carbonyl diimidazole added. After one hour at room temperature in a sealed vessel, one gram of IR45 resin, which had been dried previously under

high vacuum for three hours at 70°C, was added and the mixture stirred for eight hours in a sealed vessel. After this time, the resin was filtered off, washed three times each with 10 mls of dimethyl formamide, water and ethanol, then dried under high vacuum at 70°C. The carbobenzoxy group was removed with hydrogen bromide in acetic acid as previously described.

DL-leucyl-glycyl-DL phenylalanine was similarly reacted with weakly basic resins CG45 and AG3.

29. Amino acid analysis of substituted resins IR45, CG45 and AG3

Hydrolysis of the resins was carried out as previously described for the Merrifield resin (See 22).

A blank hydrolysis experiment on unsubstituted resin (IR45) revealed a level of impurities not much less than that expected for the chemically introduced amino acids. Lowering the temperature of hydrolysis to 70°C had no appreciable effect on the level of impurities. Resin which had already been subjected to one hydrolysis treatment was carefully washed with deionised water and rehydrolysed. Again no appreciable lowering of the impurity level was observed. Subjecting p-amino methyl toluene to identical hydrolysis conditions gave a similar but not identical impurity pattern to that obtained on hydrolysis of the unsubstituted resin.

It was concluded that the impurities present were due to chemical breakdown of the resin and thus could not be avoided. This made amino acid analysis of the substituted resins difficult. It was observed from comparing the analysis patterns of substituted and unsubstituted resins that the phenylalanine analysis was the least affected by impurities. Thus the phenylalanine analysis was taken as an indication of the amount of substitution which had taken place. Analysis for the various peptide resins are given in Table VII.

TABLE VII

Amino acid	Resin (analysis in μM per gram) IR45	CG45	AG3*
leucine	0.30	0.75	-
glycine	0.35	0.60	-
phenylalanine	0.15	0.35	-

* as no appreciable amount phenylalanine found this resin was considered to be unsubstituted.

Hence the amount of peptide coupled to the resins IR 45 and CG45 is of the order 0.1-0.4 μM per gram. Assuming that the molecular weight of the monomer unit is approximately 100, this corresponds to one monomer unit in 10^5-10^6 being substituted. The figure is also much less than the available ion exchange sites in the resin (5 m. equiv

per gram). The above seems to indicate that substitution takes place mainly on the surface of the resin.

30. Reaction of substituted resins with $Co[(triene)(OH)(OH_2)]^{++}$

1 gram of the substituted resin was suspended in 10 mls of 0.1 M solution of $Co[(triene)(OH)(OH_2)]^{++}$ and the suspension stirred for one hour at 50°C. and pH 7.5. The pH was kept at 7.5 by the dropwise addition of 0.1 M NaOH. After one hour, the resin was filtered off and washed thrice each with 10 mls of water, 10^{-3}M perchloric acid and water. At this stage the weakly basic ion exchange resins IR45 and CG45 assumed a definite orange colour. No such colour change was observed with the other resins and in these cases no reaction was considered to have taken place.

The coloured resin was suspended in 10 mls of water and the pH adjusted to 10 with 0.1 N NaOH. After one hour at room temperature the resin was filtered off and the orange eluent chromatographed on Gelman SA media, eluent BuOH:HAc:H_2O and MeOH:HAc. The process was repeated twice. Chromatography of three eluents revealed the expected amino acid complex and $Co[(triene)(OH)(OH_2)]^{++}$.

31. Reaction of IR45 and $Co[(triene)(gly)]^{++}$

1 gram of unsubstituted IR 45 resin was suspended in 10 mls of 0.05 M solution of $Co[(triene)(gly)]^{++}$ and the pH raised to 11 with 0.1 NaOH. After one hour, the

resin was filtered off and washed with 10 mls of water. The filtrate and washings were combined and applied to a small column (10 x 1 cm) of Dowex 50 x 2 resin (hydrogen form). On elution with 1M $NaClO_4$, pH 8.5 two bands separated which were identified as $Co\left[(triene)(gly)\right]^{++}$ (orange) and $Co\left[(triene)(OH)(OH_2)\right]^{++}$ (red-blue). The amount of breakdown of the glycine complex was roughly estimated from band widths as approximately 10%.

32. Reaction of IR45 and $Co\left[(triene)(leu)\right]^{++}$

The reaction was performed as described for $Co\left[(triene)(gly)\right]^{++}$. The amount of breakdown was estimated to be 10%.

33. Reaction of Dowex-1 x 10 with $Co\left[(triene)(leu)\right]^{++}$

1 gram of Dowex-1 x 10 (hydroxide form) was suspended in 10 mls of 0.01 M solution of Co (triene)(leu) $^{++}$ and the suspension warmed to 50°C for five minutes. The resin was filtered off and washed with 10 mls of water. The filtrate and washings were combined and applied to a small column of Dowex 50 x 2 (hydrogen form). On elution with 1M $NaClO_4$, pH 8.5, one band was visible which was identified as $Co\left[(triene)(OH)(OH_2)\right]^{++}$ from its colour. No orange $Co\left[(triene)(leu)\right]^{++}$ band was visible. The washed Dowex 1 x 10 resin was placed in a small column and eluted with 30 mls of 1N acetic acid. The eluent was collected,

evaporated to dryness under vacuo, the residue dissolved in a small amount of water ($\frac{1}{2}$ ml), and chromatographed on Gelman SA media, eluent BuOH:HAc:H_2O. Development with ninhydrin solution revealed leucine.

34. Reaction of ribonuclease with $Co\left[(triene)(OH)(OH_2)\right]^{++}$

(a) 21.8 mg of ribonuclease (1.6 μmoles) were dissolved in 5 mls of 0.01 M $Co\left[(triene)(OH)(OH_2)\right]^{++}$ solution and the pH adjusted to 7.5 with 2N NaOH. After two hours at 50°C the solution was cooled and passed down a short column (5 x 1 cm) of Dowex 50 x 2 (sodium form). Unreacted $Co\left[(triene)(OH)(OH_2)\right]^{++}$ was held by the resin but the large ribonuclease complex totally excluded from the resin matrix and passed through the column unhindered. The column was washed with 2 mls of water, the eluent and washings combined and made up to a volume of 10 mls with water. The optical density at 483 mμ of this orange solution was found to be 0.86. A 0.6 mole/ml solution of an amino acid complex would have a similar optical density. Thus there appear to be approximately four complex molecules per molecule of enzyme. The experiment was repeated and it was found that five molecules of complex per molecule of enzyme were present.

(b) 18.8 mg of ribonuclease (1.4 moles) were dissolved in 5 mls of solution, 0.01 M in $Co\left[(triene)(OH)(OH_2)\right]^{++}$ and 3M in $Ca(ClO_4)_2$ and the pH adjusted to 7.5 with 2N NaOH. After two hours at 50°C, the solution was cooled and the components separated as before. The optical density of the ribonuclease complex solution (10 mls) was found to be 2.52, which corresponds to eighteen molecules of complex per molecule of enzyme. The great difference in moles of complex found is quite obviously due to ribonuclease being denatured in this solvent and reaction sites in the interior being more accessible.

35. Reaction of peptides with $Co\left[(triene)(OH)(OH_2)\right]$

The peptide or peptide derivative was dissolved in a solution of $Co\left[(triene)(OH)(OH_2)\right]^{++}$ which was between 2-5 fold molar excess than the concentration of the peptide. The pH was raised to 7.5 by the dropwise addition of 0.1N NaOH and the reaction mixture heated for one hour at 50°C. After this time the mixture was lyophilised to dryness, dissolved in a small amount (1-2 mls) of 1M pyridine acetate and applied to a short column (12 x 1 cms) of carboxy methyl sephadex (pyridine form). Elution with 1M pyridine acetate separated the products (Fig 16). The eluent containing the products was lyophilised to a small volume ($\frac{1}{2}$ - 1 ml).

FIG 16

Eluent 1M pyridine acetate

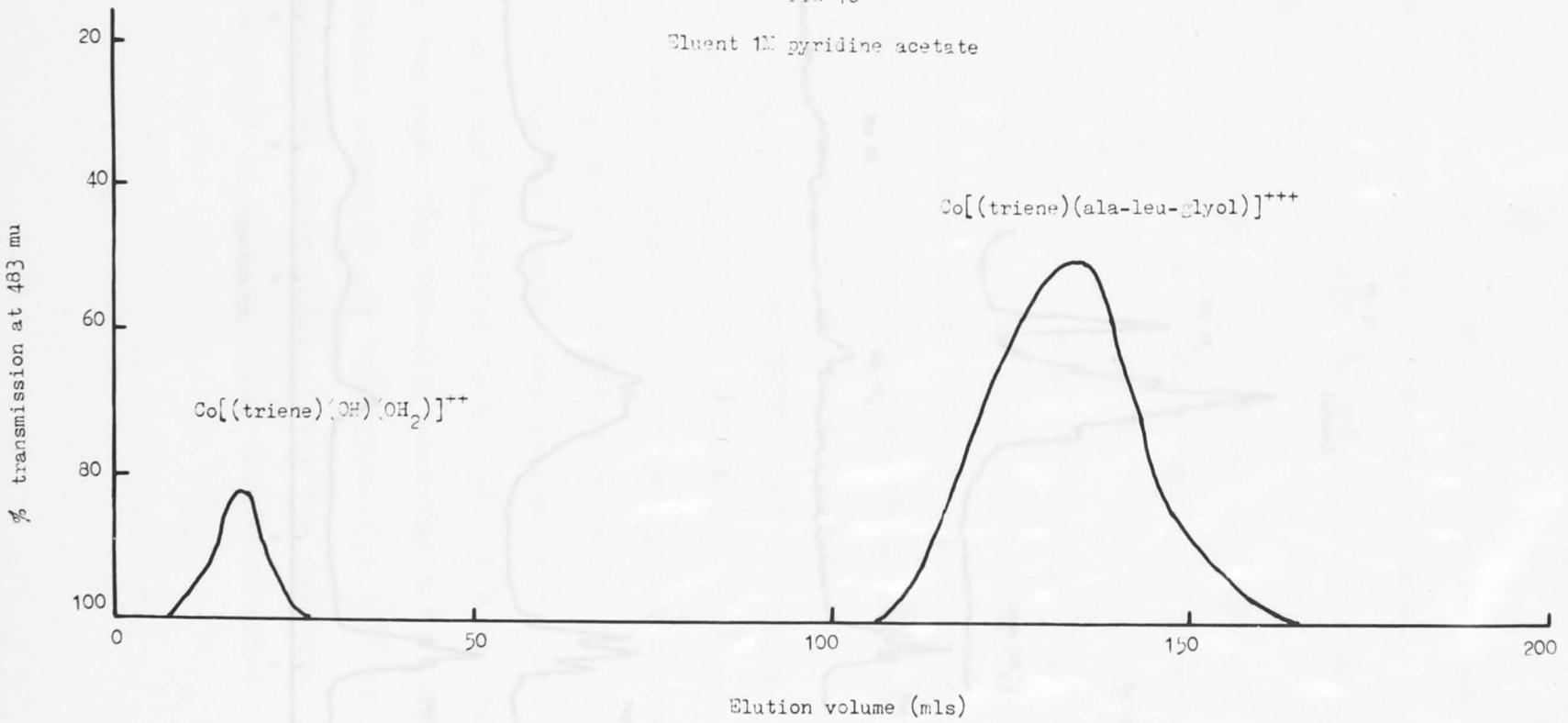
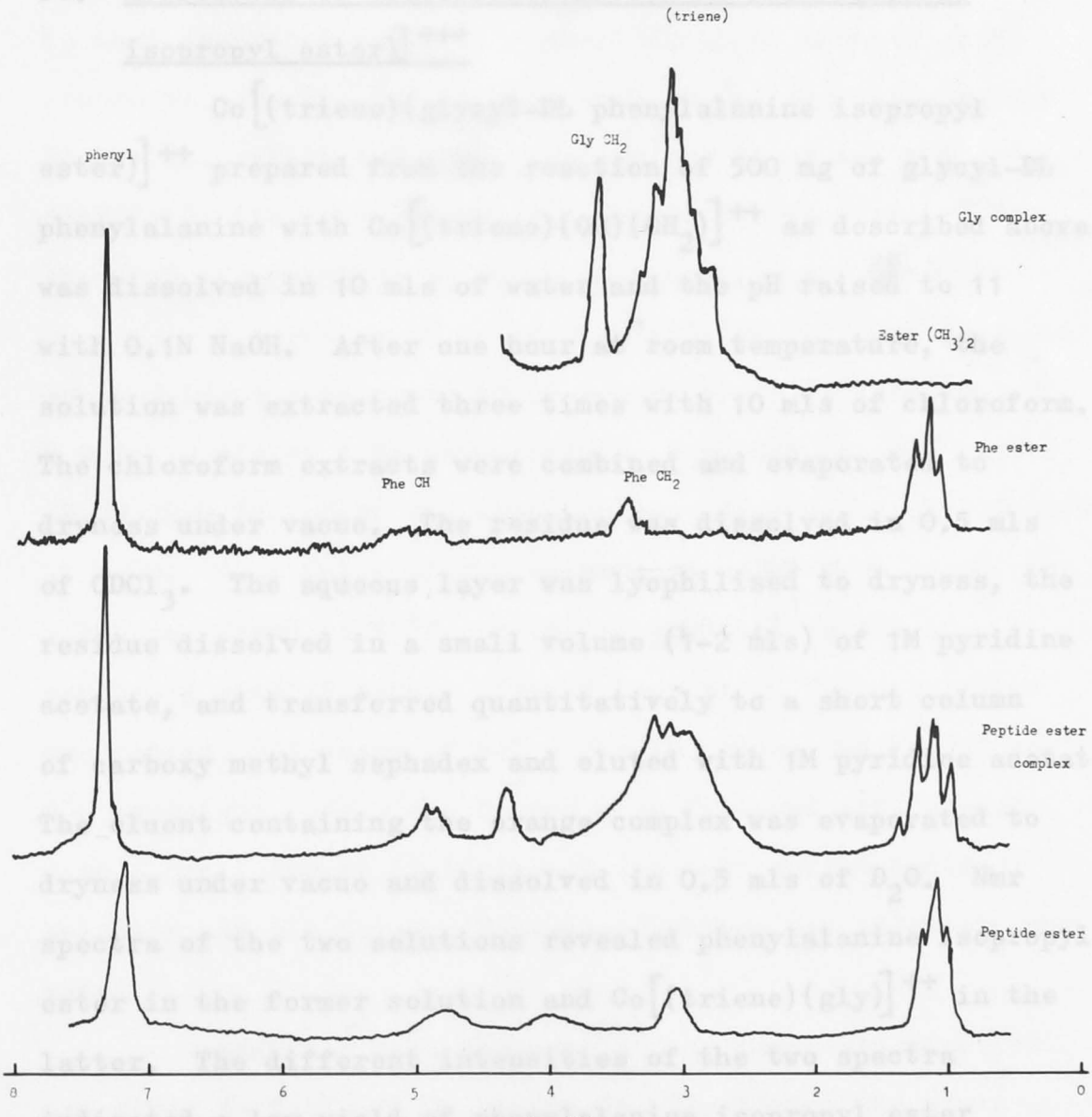


FIG 17


(triene)
Gly CH_2
phenyl
Gly complex
Ester $(CH_3)_2$
Phe ester
Phe CH
Phe CH_2
Peptide ester complex
Peptide ester
8
7
6
5
4
3
2
1
0
ppm from TMS

Solutions of peptide complexes could not be lyophilised to dryness, the end product always appearing as a gum.

36. Hydrolysis of $Co[(triene)(glycyl\text{-}DL\ phenylalanine\ isopropyl\ ester)]^{+++}$

$Co[(triene)(glycyl\text{-}DL\ phenylalanine\ isopropyl\ ester)]^{++}$ prepared from the reaction of 500 mg of glycyl-DL phenylalanine with $Co[(triene)(OH)(OH_2)]^{++}$ as described above was dissolved in 10 mls of water and the pH raised to 11 with 0.1N NaOH. After one hour at room temperature, the solution was extracted three times with 10 mls of chloroform. The chloroform extracts were combined and evaporated to dryness under vacuo. The residue was dissolved in 0.5 mls of $CDCl_3$. The aqueous layer was lyophilised to dryness, the residue dissolved in a small volume (1-2 mls) of 1M pyridine acetate, and transferred quantitatively to a short column of carboxy methyl sephadex and eluted with 1M pyridine acetate. The eluent containing the orange complex was evaporated to dryness under vacuo and dissolved in 0.5 mls of D_2O. Nmr spectra of the two solutions revealed phenylalanine isopropyl ester in the former solution and $Co[(triene)(gly)]^{++}$ in the latter. The different intensities of the two spectra indicated a low yield of phenylalanine isopropyl ester (Fig 17).

37. Hydrolysis of Co[(triene)(DL leucyl-glycyl-DL phenylalanine isopropyl ester)]$^{+++}$

The hydrolysis was performed in a similar manner to that described for Co[(triene)(glycyl-DL phenylalanine isopropyl ester)]$^{+++}$. In this case no residue was obtained in the chloroform extract. The aqueous layer was found to contain Co (triene)(OH)(OH_2) $^{++}$ and Co (triene)(leu) $^{+++}$.

38. Preparation of amino acid complexes

A solution of Co[(triene)(OH)(OH_2)]$^{++}$ was prepared by dissolving 3.84 grams (0.01 moles) of Co[(triene)CO_3] Cl. H_2O in 10 mls of 1.5M perchloric acid (0.015 moles). After 15 minutes the pH of the solution was adjusted to pH 7.5 with 2N NaOH. 0.01 moles of the amino acid ester were added, the solution warmed to 50°C and the pH adjusted to 7.5 if necessary with 2N NaOH. After 1 hour at 50°C the solution was cooled, 0.5 grams of NaI or $NaClO_4$ added and stored at 0°C overnight. Crystals formed were filtered off, washed with a small amount (2-5 mls) of ice cold sodium iodide solution and air dried. Recrystallisation was effected from water.

Analytical data for some of the complexes prepared are given in Table VIII. Table IX illustrates the yield (in grams) obtained from the reaction of 0.01 moles of amino acid ester with 0.01 moles of Co[(Triene)CO_3] Cl.H_2O.

TABLE VIII

ANALYTICAL DATA FOR COMPLEXES

Compound	Calculated			Found		
	C	H	N	C	H	N
$Co[(triene)(phe)]I_2.1.5H_2O$	27.70	10.78	4.77	27.68	10.70	4.70
$Co[(triene)(val)]I_2.2H_2O$	21.61	11.46	5.24	21.34	11.22	5.13
$Co[(triene)(leu)]I_2.2H_2O$	23.42	11.39	5.20	23.48	11.22	5.56
$Co[(triene)(arg)]I_3.1.5H_2O$	18.33	14.25	4.20	18.86	13.99	4.68
$Co[(triene)(lys)](ClO_4)_3.1.5H_2O$	21.29	12.43	5.06	21.28	12.39	4.90
$Co[(triene)(cyst.acid)]ClO_4.1.5H_2O$	21.70	14.07	5.22	21.68	14.04	5.07

TABLE IX

YIELD OF COMPLEXES FROM 0.01 MOLES OF ESTER

Complex of	Yield (grms)
Arginine	3.4
Serine	1.60
Lysine	3.84
Aspartic acid	0.95
Cysteic acid	2.7
Cystein (a)	-
Glycine	1.5
Valine	1.27
Phenylalanine	1.30
Leucine	1.5
Histidine	0.65
Alanine	1.0
Isoleucine	1.9
Tyrosine	3.4
Proline (b)	-

(a) black ppte formed, presumably CoS.

(b) could not be crystallised.

39. Hydrolysis of amino acid esters

A solution of the amino acid ester (10% w/v) and sodium carbonate (0.1N) in 2 mls of D_2O was prepared by weighing out the calculated amounts into a small beaker (10 mls) and adding 2 mls of D_2O. The pH was raised to 10.5 with 2N NaOD and 1 ml of this solution quickly transferred to a nmr spectrometer tube. The tube was placed in the spectrometer as soon as possible after preparation of the solution and scanned at predetermined time intervals. The time of reaction was taken as the time at which the spectrometer scanned across the ester proton peak. Zero time was taken as the time at which the pH was raised to 10.5

40. Reaction of glycyl-glycine isopropyl ester hydro perchlorate with $Co\left[(triene)(OH)(OH_2)\right]^{++}$

279 mg of glycyl-glycine isopropyl ester hydro perchlorate (1.0 m moles) were weighed out into a small beaker (10 mls) and 10 mls of 0.01M solution of $Co\left[(triene)(OH)(OH_2)\right]^{++}$, (0.1 m moles), pH 3.0 added. The pH was then raised to 7.5 with 1N NaOH and 3 mls of this solution immediately transferred to a 1 cm quartz spectrophotometer cell. Visible spectra were obtained at predetermined times on a Carey 14 spectrophotometer and the

optical density at 483 mμ and 505 mμ determined. Zero time for the reaction was taken as the time at which the pH was raised to 7.5. The results are shown in Table X.

TABLE X

Time (mins)	D_{483}	D_{505}
4.75	1.3025	1.4650
10.08	1.3475	1.4550
15.92	1.3850	1.4500
20.50	1.4050	1.4400
25.16	1.4100	1.4375
31.75	1.4375	1.4325
35.92	1.4500	1.4175
40.16	1.4575	1.4175
44.50	1.4625	1.4150
52.08	1.4750	1.4100
56.16	1.4800	1.4102

41. Reaction of DL alanyl-DL leucyl-glycinol with $Co\left[(triene)(OH)(OH_2)\right]^{++}$

(a) 10 mls of solution, 0.1M in DL alanyl-DL leucyl-glycinol and 0.01M in $Co\left[(triene)(OH)(OH_2)\right]^{++}$ was prepared as described for the reaction of glycyl-glycine isopropyl ester hydroperchlorate in procedure 40, the pH raised to 7.5

and the optical density of the solution determined at various times. No visible change in spectra was observed after 24 hours reaction at room temperature.

(b) 1 ml of solution 1M in DL alanyl-DL leucyl-glycinol and 1M in $Co\left[(triene)(OH)(OH_2)\right]^{++}$ was prepared as described in procedure 40. The pH was raised to 7.5 and the solution placed in a water bath at 60°C. At predetermined times, 0.1 ml samples were withdrawn using a micrometer syringe and diluted to 10 mls. The optical densities at 483 mμ and 505 mμ were determined as before (procedure 40). Results are shown in Table XI.

TABLE XI

Time (hours)	D_{483}	D_{505}
1.0	1.332	1.540
2.5	1.285	1.400
5.0	1.305	1.340
8.0	1.32	1.255
14.0	1.15	1.015

42. Hydrolysis of Co (triene)(DL ala-DL leu-glycinol) $^{++}$

$Co\left[(triene)(DL\ ala\text{-}DL\ leu\text{-}glycinol)\right]^{+++}$ was prepared from 2 grams of DL ala-DL leu-glycinol as previously described (procedure 35). The product was dissolved in 25 mls of water and stored at 4°C.

5 mls of this solution was pipetted into a small beaker (10 mls) and placed in a water bath at the temperature of the reaction. After allowing time for equilibration (15 mins), the pH was raised to the required value with 1M NaOH. After a predetermined time, the reaction was quenched by lowering the pH to 4.5 with the addition of 1M acetic acid. The solution was removed from the water bath, lyophilised to dryness and dissolved in a small amount of eluent (1-2 mls). The solution was transferred quantitatively to a small column (12 x 1 cm) of carboxy methyl sephadex and eluted with either 1M pyridine acetate or a 0.1 - 0.3 M gradient of ammonium bicarbonate pH 10.0. The eluent containing the various coloured bands was collected, lyophilised to dryness and dissolved in 5 mls of water. The optical density of the solutions was determined at 483 mμ and room temperature (see Tables XVII, XVIII).

IDENTIFICATION OF AMINO ACID COMPLEXES

(a) Chromatography

Chromatography was conducted on a series of different media using either ascending or descending techniques in a sealed glass tank at room temperature. Media were spotted with 1-2 μl of 0.01 M solutions using a micropipette and developed with ultraviolet light in the case of amino acid complexes or a 1% solution of ninhydrin in alcohol for amino acids. It is worth noting that amino acid complexes are ninhydrin positive although this was not used in developing amino acid complex chromatograms.

R_F values for the various media and solvent systems used are given in Tables XII, XIII, XIV. Errors, where stated, were obtained from two or more measurements.

A method frequently used for identifying amino acid complexes was chromatography of the free amino acid obtained from the reaction of a strongly basic ion exchange resin and the amino acid cobalt complex.

TABLE XII*

Complex of	R_F (a)	R_F (b)	R_F (c)
Leucine	1.00	1.00	1.00
Phenylalanine	0.98	0.96 ± .01	0.96 ± .04
Valine		0.76 ± .01	0.76 ± .02
Glycine	0.30	0.29 ± .01	0.58 ± .05
Cysteic acid		0.20 ± .01	0.49 ± .02
Arginine	0.54	0.30 ± .01	0.36 ± .05
Lysine	0.42	0.21 ± .01	0.38
Histidine			0.39 ± .01
Serine			0.77 ± .03
Aspartic acid			0.47 ± .02
Alanine			0.64

* Eluent BuOH:H_2O:HAc 100:100:20

(a) ascending thin layer chromatography on cellulose

(b) descending chromatography on Whatman 3M paper

(c) ascending chromatography on Gelman ITLC SA media

TABLE XIII*

Complex of	R_F (a)	R_F (b)	R_F (c)
Leucine	1.0	1.0	1.0
Phenylalanine	0.97	0.65 ± .02	0.80 ± .03
Valine	0.87	0.58 ± .02	0.78 ± .03
Glycine	0.69	0.57 ± .02	0.64 ± .04
Cysteic acid	0.44	0.36 ± .02	0.46 ± .02
Arginine	0.63	0.44 ± .02	0.58 ± .03
Lysine	0.57	0.44 ± .02	0.54 ± .03
Histidine	0.70	0.41 ± .02	0.57 ± .03
Aspartic acid	0.86	0.91 ± .02	
Serine	0.88	0.83 ± .02	
Alanine	0.91	0.95 ± .01	
Isoleucine	1.06	0.79 ± .03	0.93 ± .05
Tyrosine	1.47	2.85 ± .15	

* Eluent MeOH:HAc 10:1

(a) ascending chromatography on Gelman ITLC SA media

(b) ascending chromatography on buffered Gelman ITLC SA media (pH 10.5)

(c) ascending chromatography on buffered Gelman ITLC SA media (pH 4.0)

TABLE XIV*

Amino acid	R_F(a)	R_F(b)	R_F(c)
Alanine	0.92 ± .01		0.67 ± .01
Arginine		0.48 ± .01	0.28 ± .01
Aspartic acid			0.36 ± .01
Cysteic acid			0.31 ± .01
Glycine	0.87 ± .01	0.72 ± .01	0.59 ± .01
Glutamic acid			0.52 ± .01
Histidine		0.50 ± .01	0.65 ± .01
Isoleucine	1.00 ± .01		0.90 ± .01
Leucine	1.01 ± .01	0.97 ± .01	0.89 ± .01
Lysine		0.44 ± .01	0.18 ± .01
Phenylalanine	1.00	1.00	1.00
Proline	0.75 ± .01	0.60 ± .01	0.92 ± .01
Serine		0.73 ± .01	9.55 ± .01
Tryptophan			1.05 ± .01
Tyrosine		0.97 ± .01	0.88 ± .01
Valine		0.87 ± .01	0.70 ± .01

* ascending chromatography on Gelman ITLC SA media

(a) eluent MeOH:HAc 10:1

(b) eluent BuOH:HAc:H_2O 6:2:2

(c) eluent Phenol:H_2O 4:1

(b) Visible and near ultra violet spectroscopy

Spectra were obtained on a Carey 14 spectrometer at room temperature using a scanning speed of 5A^{o} per minute and solutions approximately 0.01 M in complex. It is seen that the spectra are very similar and offer no method for unique characterisation of amino acid complexes. Table XV gives the maximum extinction coefficient for several complexes.

TABLE XV

Complex	λmax	E
Valine	480	155
phenylalanine	481	140
Leucine	470	148
Gly-gly-O isopropyl	483	155
Co (triene)(OH)$(OH_2)^{++}$	505	185
lysine	476	144

Fig 18 shows that at least for the lysine complex Beers law is obeyed.

(c) Nuclear magnetic resonance spectra

Nuclear magnetic resonance spectra of several amino acid complexes are illustrated in Fig. 19. The broad peak at approximately 7.5-8.0 tau can be assigned to the protons of the triene group present in the complex.

FIG 18

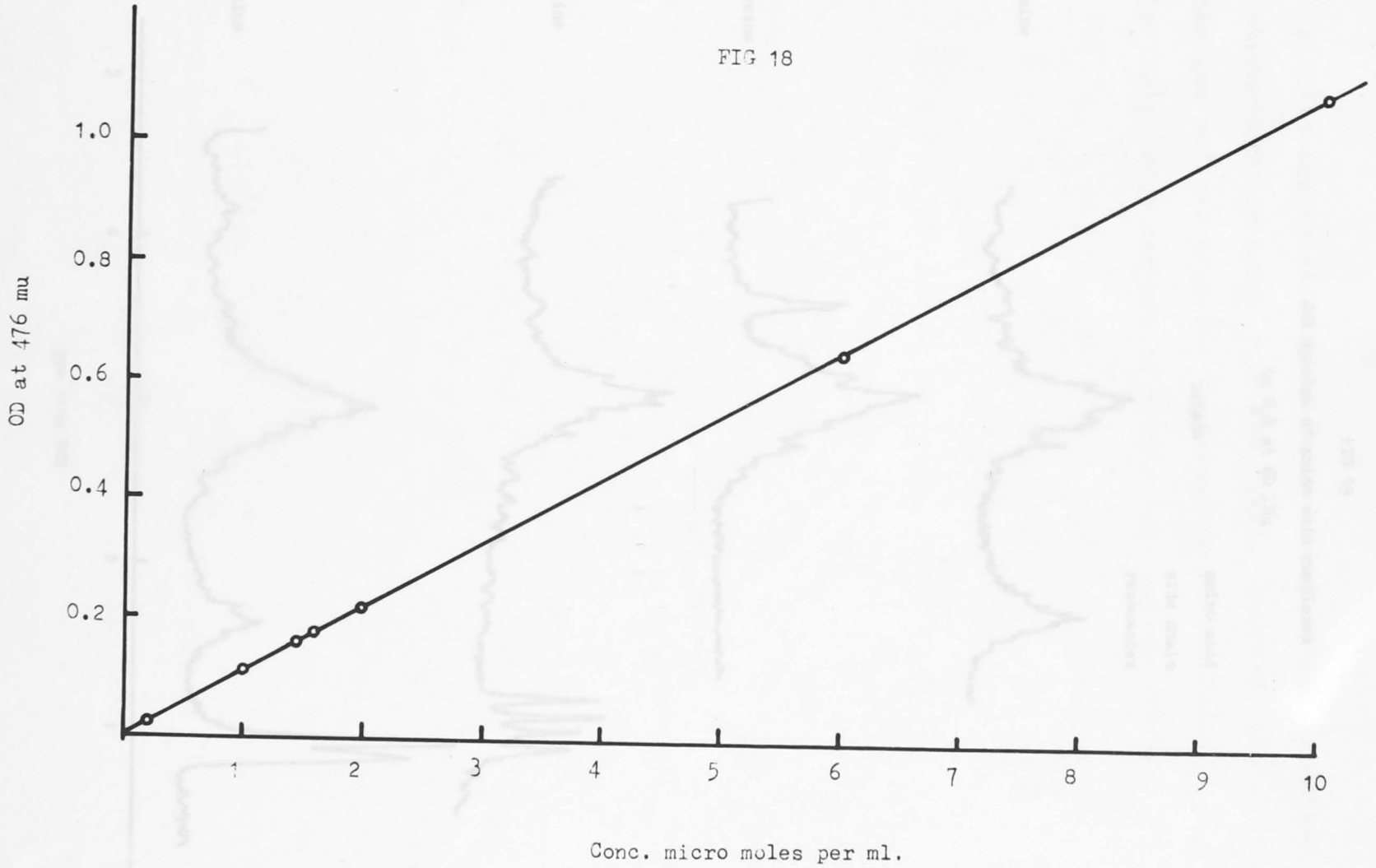
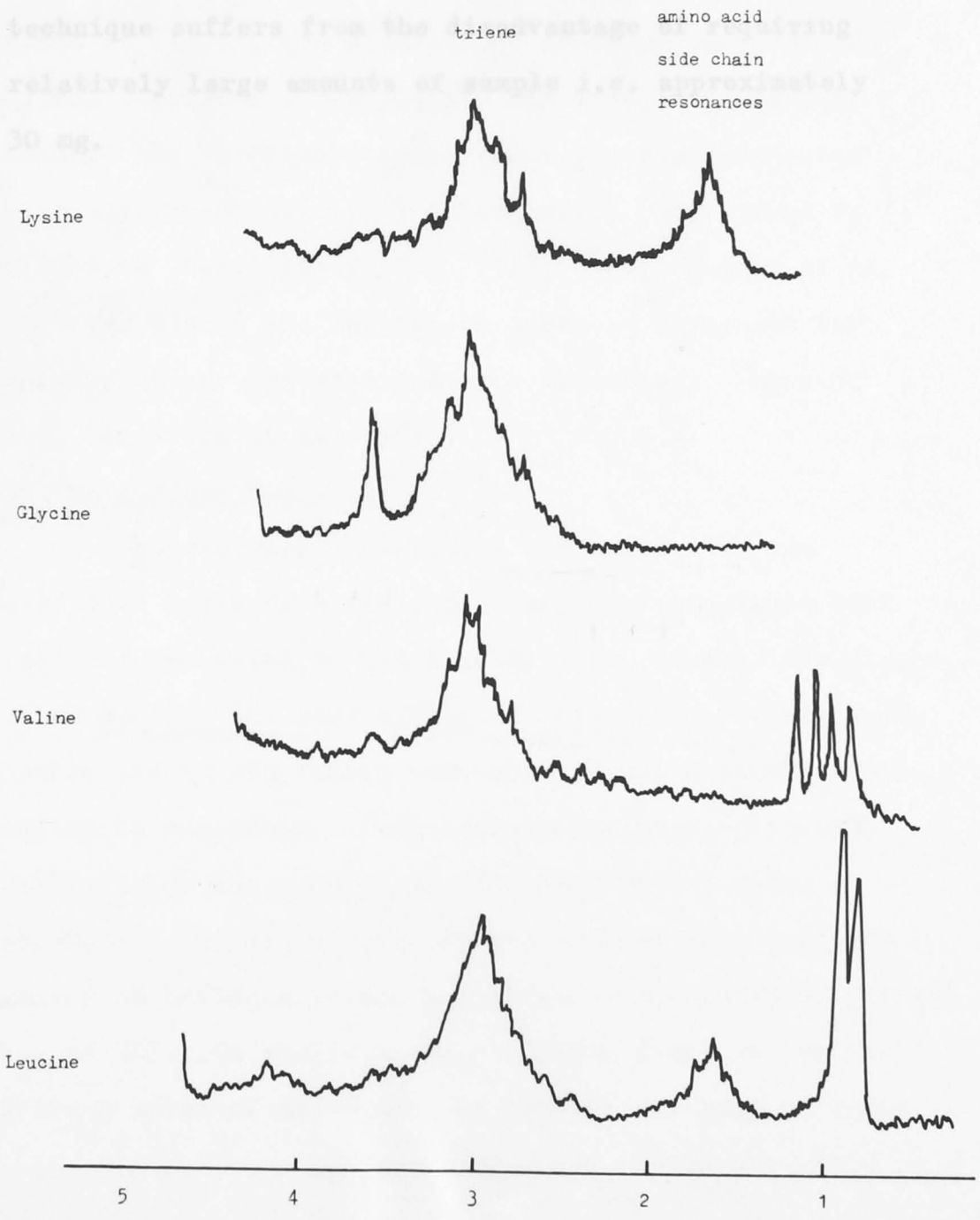


FIG 19

NMR spectra of amino acid complexes in D_2O at 60 MHz



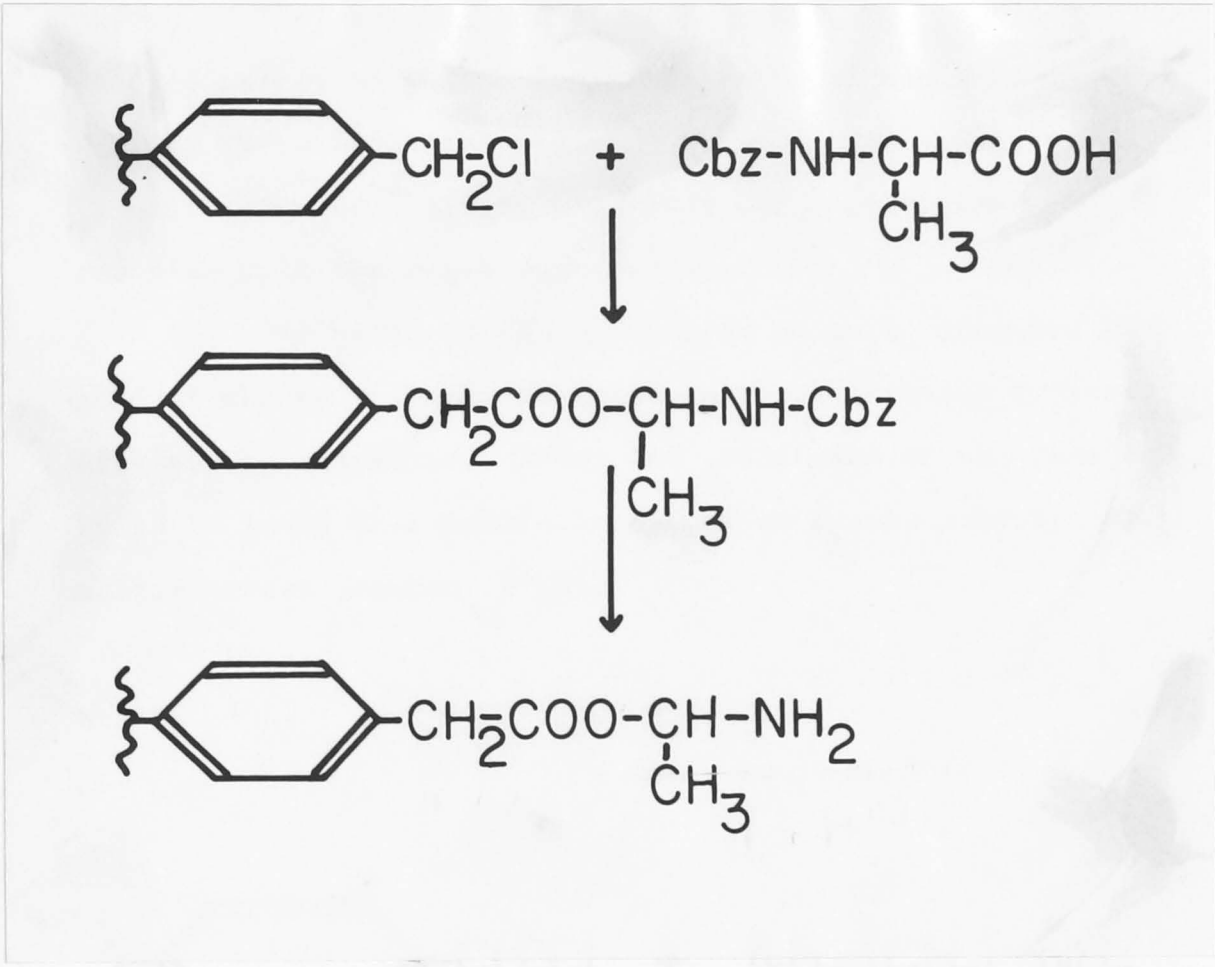
Nmr spectra are considered to be the least ambiguous method of characterising amino acid complexes. However, the technique suffers from the disadvantage of requiring relatively large amounts of sample i.e. approximately 30 mg.

POLYMER SUPPORTS

In order to facilitate the separation of III and IV of Fig 15, the possibility of using an insoluble polymer support for the peptide has been investigated. Although the Merrifield process for peptide synthesis using a polymer support of chloromethyl polystyrene is well known (Merrifield, 1962, 1963, 1964; Berger et al, 1970) the use of the Merrifield resin as a support for degradation has not received much attention. (Lawson, 1966; Dijkstra et al; 1967).

(a) Merrifield Resin

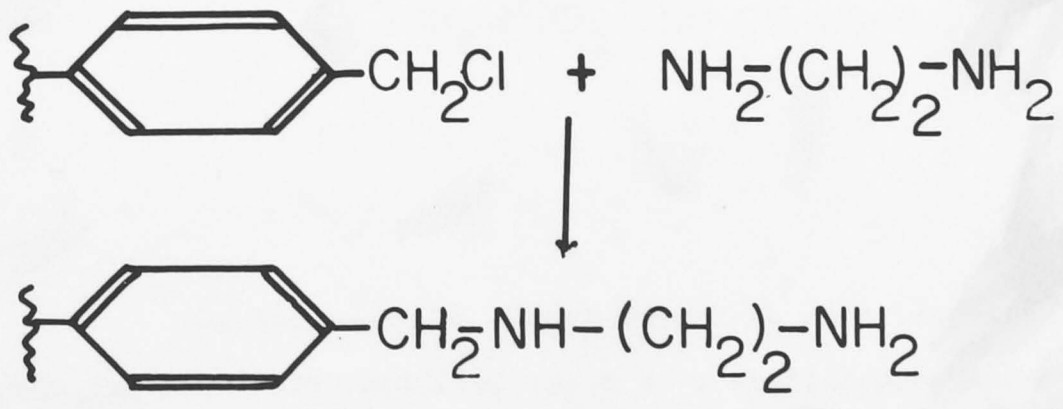
Carbobenzoxy-L-alanine was coupled to the Merrifield resin by refluxing a sample of the resin with carbobenzoxy-L-alanine and triethylamine in absolute alcohol for 60 hours. The same number of moles of carbobenzoxy-L-alanine and triethylamine were used as moles of chlorine present in the resin. Carbobenzoxy-DL leucyl-glycyl-DL phenylalanine was coupled to the resin by a similar procedure. The blocking group was removed with hydrogen bromide in acetic acid and a portion of the resin hydrolysed with 6N HCl. On analysis the resin was found to contain 50-100 u moles of amino acid or peptide per gram of resin.



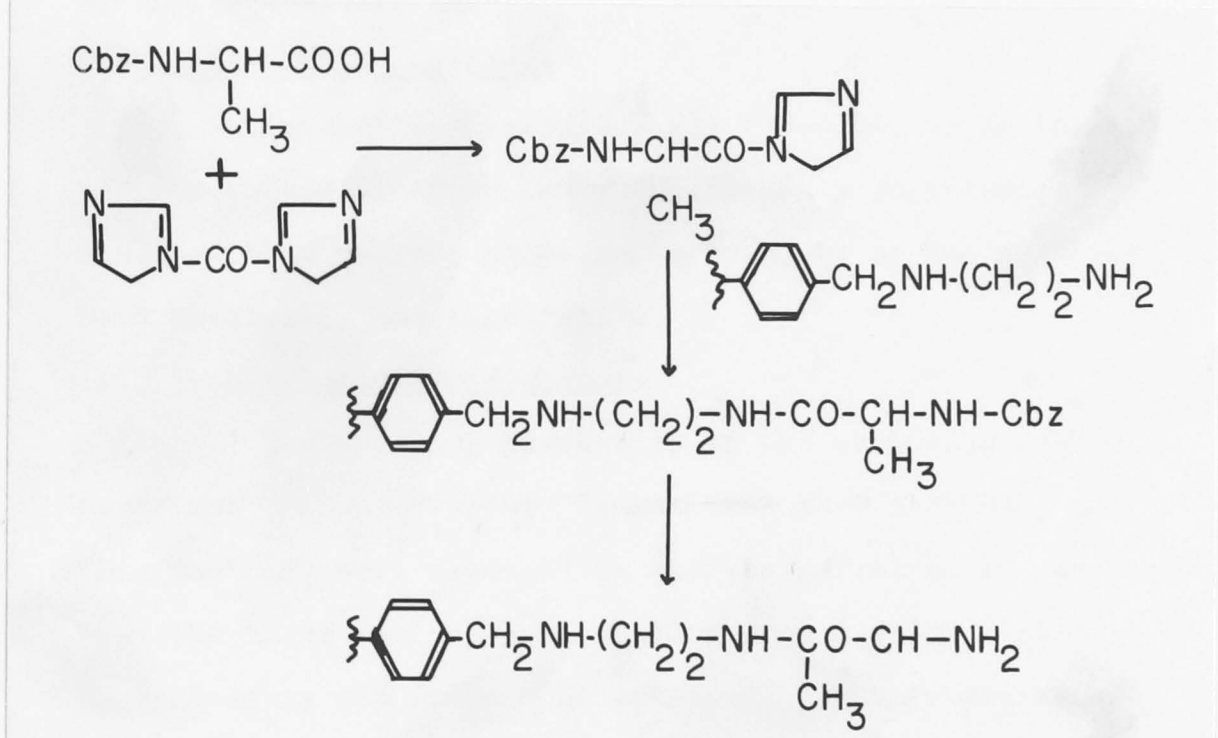
However, attempts to react the free amino group of the peptide in aqueous solution were unsuccessful. Microscopic examination of the resin beads in the coupling media, ethyl alcohol, and the reaction media, water at pH7, revealed that the swelling properties of the resin in the two media were very different. The resin was found to be highly swollen in the coupling media but contracted in the reaction media. It is felt that due to the highly swollen

nature of the resin in the coupling media, the peptide is coupled mainly to groups in the interior of the resin. On placing the substituted resin in the reaction media, water, it contracts, effectively shielding the peptide from reaction with the sequenating agent.

The resin was then modified so as to lengthen the pendant chains and make the resin more hydrophilic by reaction with ethylene diamine. Under the conditions of the reaction it is believed that little crosslinking of the polystyrene matrix occurs (Lawson, 1966).



Carbobenzoxy-L-alanine was coupled to the modified resin using carbonyl diimidazole (Anderson and Paul, 1958, 1960) in anhydrous tetrahydrofuran.



Carbobenzoxy-DL leucyl-glycyl-DL phenylalanine was coupled to the modified resin by a similar procedure. The blocking group was removed, as before, by the use of hydrogen bromide in acetic acid. Although analysis of the resin revealed that an appreciable amount (50μmoles per gram of resin) of the amino acid or peptide had been

substituted on the resin, no reaction with $Co\left[(triene)(OH)(OH_2)\right]^{++}$ was apparent. Again, it was found that the resin was highly swollen in the coupling media, but contracted in the reaction media effectively shielding the coupled peptide from attack by the sequenating agent.

(b) Amino ethyl cellulose

Amino ethyl cellulose was found to couple the peptide carbobenzoxy-DL leucyl-glycyl-DL phenylalanine. However, this polymer support was found to be too unstable, both chemically and physically.

(c) Weakly basic ion exchange

The swelling properties of the following weakly basic ion exchange resins, "Amberlite" IR45 and CG45, and "Bio Rad" AG3 were examined in various solvents. It was found that the three resins were expanded from the dry state approximately 10% in each of water, dimethyl formamide and 0.1 N HCl and 100% in anhydrous tetrahydrofuran. Therefore, dimethyl formamide instead of tetrahydrofuran was used as the coupling media. Carbobenzoxy-DL leucyl-glycyl-DL phenylalanine was coupled to "Amberlite" IR45 andCG 45 resins using carbonyl diimidazole. No peptide was observed to couple to "Bio Rad" AG3.

Presumably, the basic properties of this resin result from groups other than the primary amine groups required for reaction with carbonyl diimidazole (e.g. tertiary amine groups).

Table XVI gives the observed yields of peptide coupled to the resins and the calculated yields based on substitution at the surface of the resin bead only. (See Appendix B).

TABLE XVI

Resin Type	Mesh	Calc. yield %	Obs. yield %
IR45	50	2×10^{-4}	$(10^{-3} - 10^{-4})$
CG 45	200	8×10^{-4}	
AG3	200	-	NIL

The similar values for the observed and calculated yields seem to indicate that substitution has taken place at the surface of the resin bead.

After reaction with $Co\left[(triene)(OH)(OH_2)\right]^{++}$ the resin was observed to become a bright orange colour which could not be removed by washing with water or dilute perchloric acid (1×10^{-3}M). Reaction of the coloured resin at pH 11 yielded an orange solution. Chromatography identified the substance as the cobalt complex of leucine. Subsequently, both glycine and phenylalanine complexes were removed separately from the resin and identified by chromatography.

However, the procedure was not reproducible.

Experiments using the unsubstituted resin and $Co\left[(triene)(gly)\right]^{++}$ or $Co\left[(triene)(Leu)\right]^{++}$ showed that the resin had a marked effect on the complex. After contact with unsubstituted resin at pH 11 for 60 minutes both complexes exhibited a marked decomposition into $Co\left[(triene)(OH)(OH_2)\right]^{++}$ and the free amino acid. Furthermore, the resin has undesirable effects on the peptide coupled to it. Storage of the resin, to which peptide had been coupled, either in the dry or wet state for several days resulted in complete destruction of the peptide.

Analysis of such resins after two or three days storage showed complete absence of amino acids. What exactly happens to the amino acids is a matter for conjecture. Catalytic decomposition of amino acid complexes is further shown by strongly basic resins such as "Dowex 1" when decomposition to the $Co\left[(triene)(OH)(OH_2)\right]^{++}$ and the free amino acid occurred within minutes at $50^{o}C$. The $Co\left[(triene)(OH)(OH_2)\right]^{++}$ remains in solution whereas the free amino acid is absorbed onto the resin and may later be eluted by 1M acetic acid and characterised.

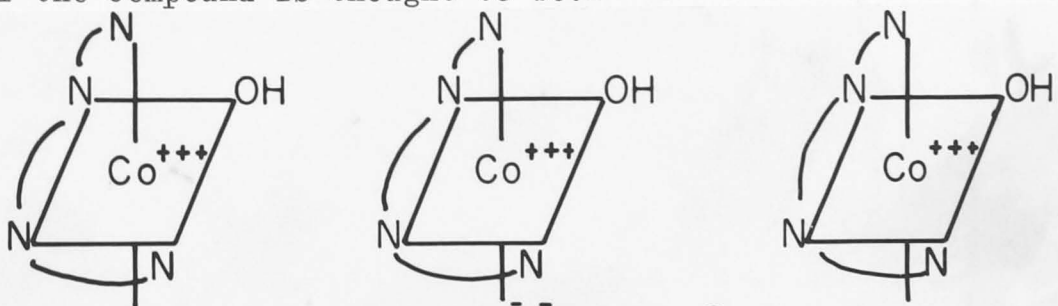
It is felt that because of the basic nature of the resins, the high concentration of free amino groups in the vicinity of the peptide or peptide complex results in a high local pH which destroys the peptide or peptide complex.

Because of the problems associated with swelling properties and high local pH conditions, it is felt that the resins investigated do not have the necessary characteristics required for a polymer support. An ideal solid support should contain relatively few free reaction sites which are widely spaced in the polymer matrix, be equally swollen in the coupling media and reaction media, be mechanically and chemically stable and have a large pore space to enable easy penetration by peptide molecules. As yet no satisfactory support has been found. A possible support is either low cross-linked poly-acrylamide gel which contains some free amino groups or a copolymer of vinyl alcohol and vinyl amine.

REACTION OF CARBOXYLS WITH $Co\left[(triene)(OH)(OH_2)\right]^{++}$

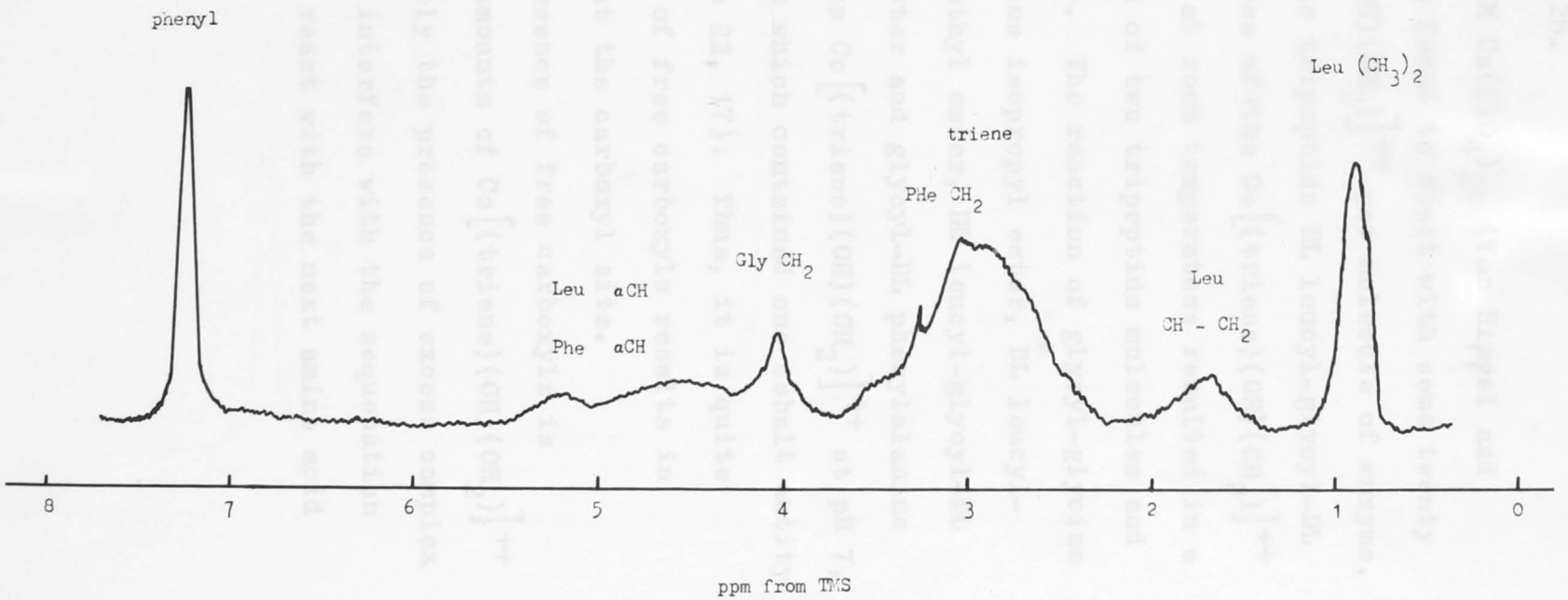
An orange product was isolated from the reaction of the free tripeptide DL leucyl-glycyl-DL phenylalanine with $Co\left[(triene)(OH)(OH_2)\right]^{++}$ at pH 7. The triene group of the cobalt entity contains twelve protons which have a resonance at 6.5-7.5 tau. By comparing the area under this peak with the area under a discrete peak due to protons contained in the peptide e.g. aromatic protons of phenylalanine or side chain protons of leucine, the ratio of cobalt to peptide can be obtained.

Examination of the nmr spectrum of the product (Fig 20) showed that the compound was composed of three cobalt entities to two tripeptide molecules. The structure of the compound is thought to be:-



Ribonuclease when reacted with a large excess of complex at pH7 resulted in a product which was estimated from optical density measurements to contain between four and five molecules of complex. When the reaction was carried out

FIG 20


phenyl
Leu aCH
Phe aCH
Gly CH_2
triene
Phe CH_2
Leu
CH - CH_2
Leu $(CH_3)_2$
8
7
6
5
4
3
2
1
0
ppm from TMS

in a denaturing solvent, 3M $Ca(ClO_4)_2$, (Von Hippel and Wong, 1964) the enzyme was found to react with some twenty molecules of $Co\left[(triene)(OH)(OH_2)\right]^{++}$ per molecule of enzyme.

Reacting the free tripeptide DL leucyl-glycyl-DL phenylalanine with an excess of the $Co\left[(triene)(OH)(OH_2)\right]^{++}$ at pH 11 for several days at room temperature resulted in a complex which was composed of two tripeptide molecules and one cobalt entity (Fig 21). The reaction of glycyl-glycine methyl ester, glycyl-glycine isopropyl ester, DL leucyl-glycyl-DL phenylalanine methyl ester, DL leucyl-glycyl-DL phenylalanine isopropyl ester and glycyl-DL phenylalanine isopropyl ester with excess $Co\left[(triene)(OH)(OH_2)\right]^{++}$ at pH 7, each resulted in a complex which contained one cobalt entity per peptide molecule (Figs 22, 17). Thus, it is quite obvious that the presence of free carboxyls results in absorption of the cobalt at the carboxyl site.

Although the presence of free carboxyls is undesirable due to large amounts of $Co\left[(triene)(OH)(OH_2)\right]^{++}$ which are absorbed, evidently the presence of excess complex at the higher pH does not interfere with the sequenation reaction i.e. it does not react with the next amino acid along the peptide chain.

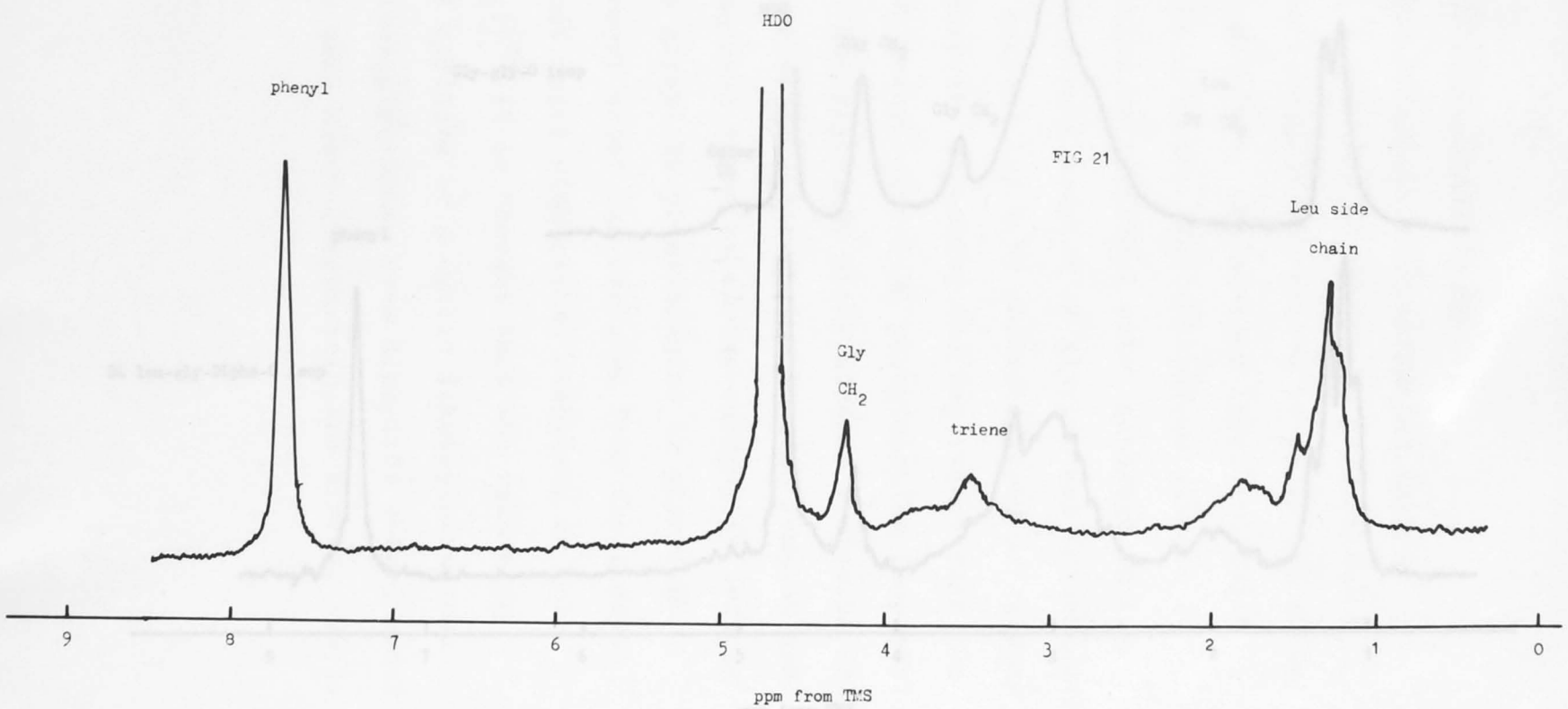
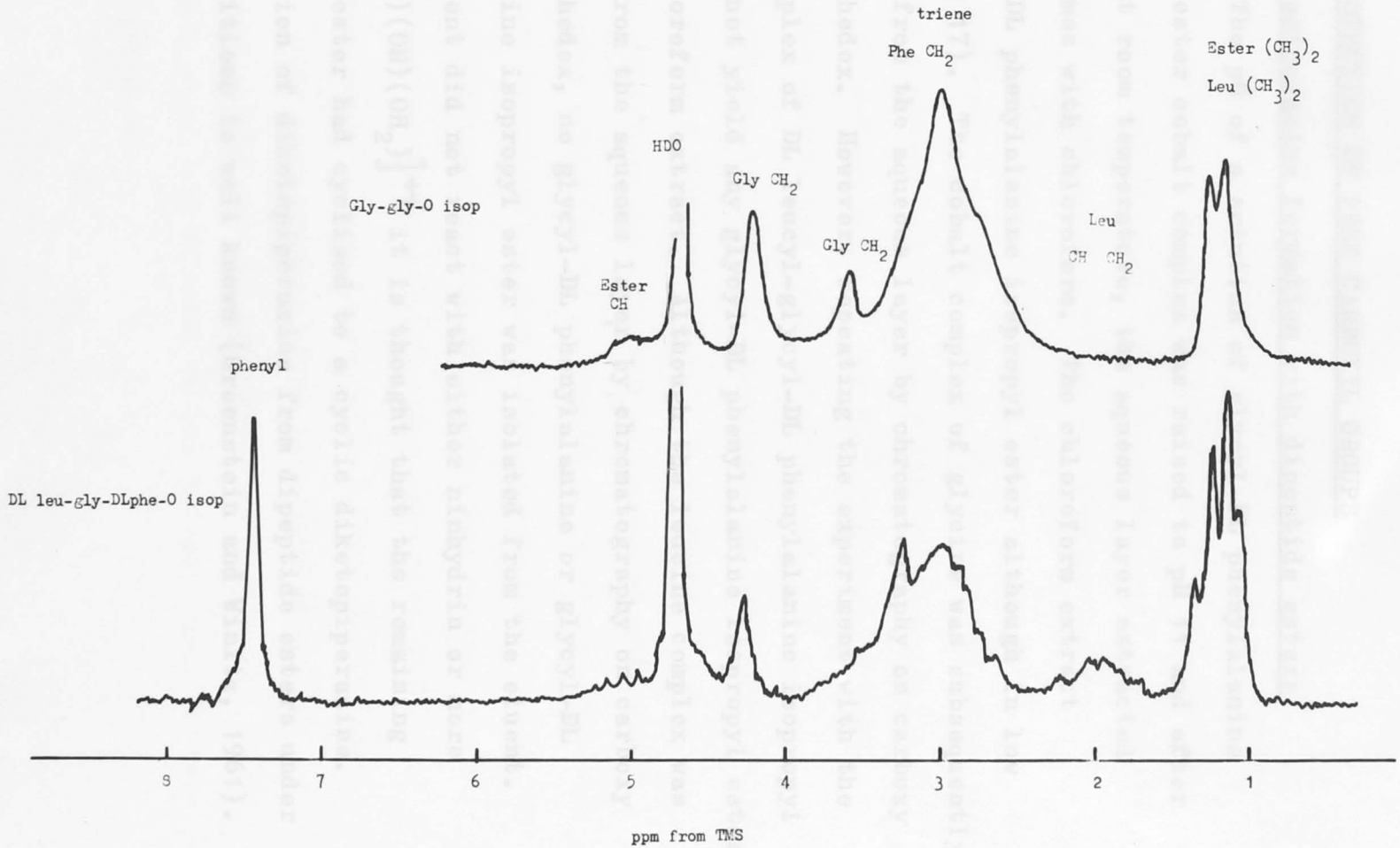

HDO
phenyl
Gly
CH_2
triene
Leu side
chain
9
8
7
6
5
4
3
2
1
0
ppm from TMS

FIG 21

FIG 22

Cobalt complexes of peptide esters



PROTECTION OF FREE CARBOXYL GROUPS

(a) Diketopiperazine formation with dipeptide esters

The pH of a solution of glycyl-DL phenylalanine isopropyl ester cobalt complex was raised to pH 11 and after one hour at room temperature, the aqueous layer extracted several times with chloroform. The chloroform extract contained DL phenylalanine isopropyl ester although in low yield (Fig 17). The cobalt complex of glycine was subsequently separated from the aqueous layer by chromatography on carboxy methyl Sephedex. However, repeating the experiment with the cobalt complex of DL leucyl-glycyl-DL phenylalanine isopropyl ester did not yield any glycyl-DL phenylalanine isopropyl ester in the chloroform extract. Although the leucine complex was isolated from the aqueous layer by chromatography on carboxy methyl sephedex, no glycyl-DL phenylalanine or glycyl-DL phenylalanine isopropyl ester was isolated from the eluent. As the eluent did not react with either ninhydrin or more $Co\left[(triene)(OH)(OH_2)\right]^{++}$ it is thought that the remaining dipeptide ester had cyclised to a cyclic diketopiperazine. The formation of diketopiperazine from dipeptide esters under these conditions is well known (Greenstein and Winitz, 1961).

(b) Stability of amino acid esters

The stability of amino acid esters in aqueous solutions of high pH has been investigated using nmr spectroscopy. The hydrolysis reaction may be followed by observing the disappearance of the resonance peak due to the ester protons or the appearance of the resonance due to protons of the liberated alcohol.

The methyl or ethyl esters of leucine, phenylalanine, tyrosine and arginine are completely hydrolysed within a few minutes at pH 10.5 as shown by the disappearance of the resonance peak due to the ester protons.

However, the rate of hydrolysis of the methyl ester of cysteic acid is somewhat slower. The hydrolysis was followed by observing the decrease in height of the methyl ester resonance (Figs 23, 24). From Fig. 24 it is estimated that complete hydrolysis occurs within ten hours. As the peak height is proportional to the area under the resonance and hence the concentration, a first order rate plot of log (peak height) against time is linear. A first order rate of 5×10^{-2} min^{-1} was obtained from the slope (Fig 25). Probably, the slower rate of hydrolysis is due to the electrostatic repulsion offered to the hydroxyl ions by the SO_3^- group present.

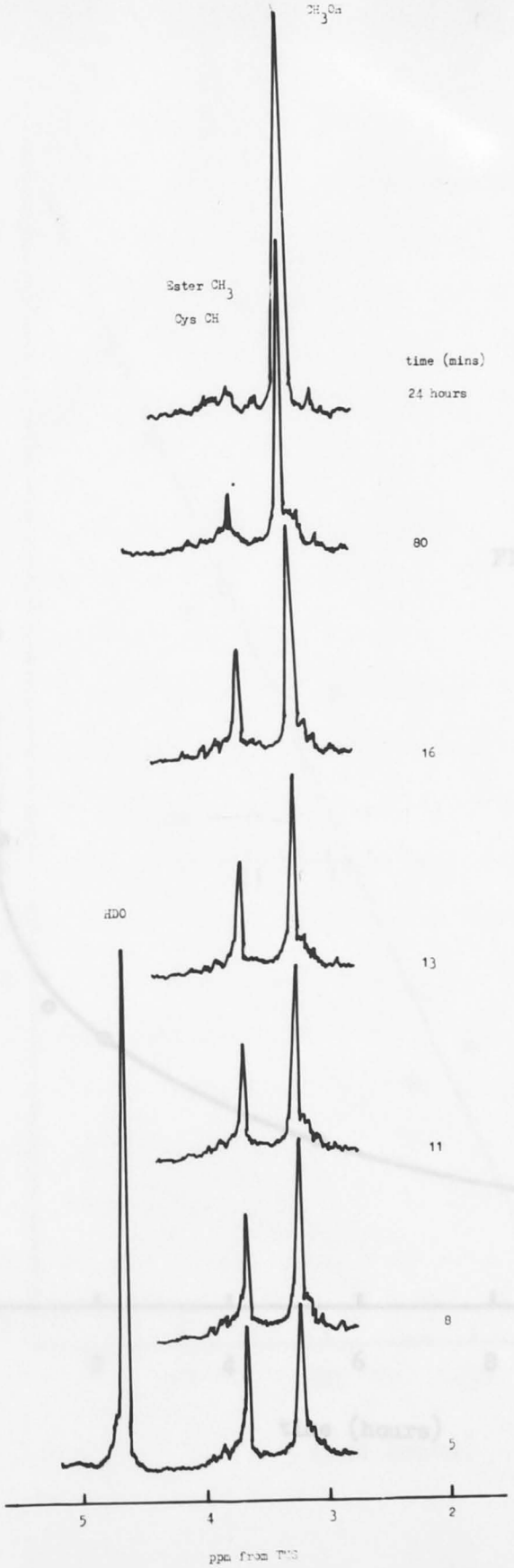

CH_3Cn
Ester CH_3
Cys CH
time (mins)
24 hours
80
16
HDO
13
11
8
5
5
4
3
2
ppm from TMS

FIG 23

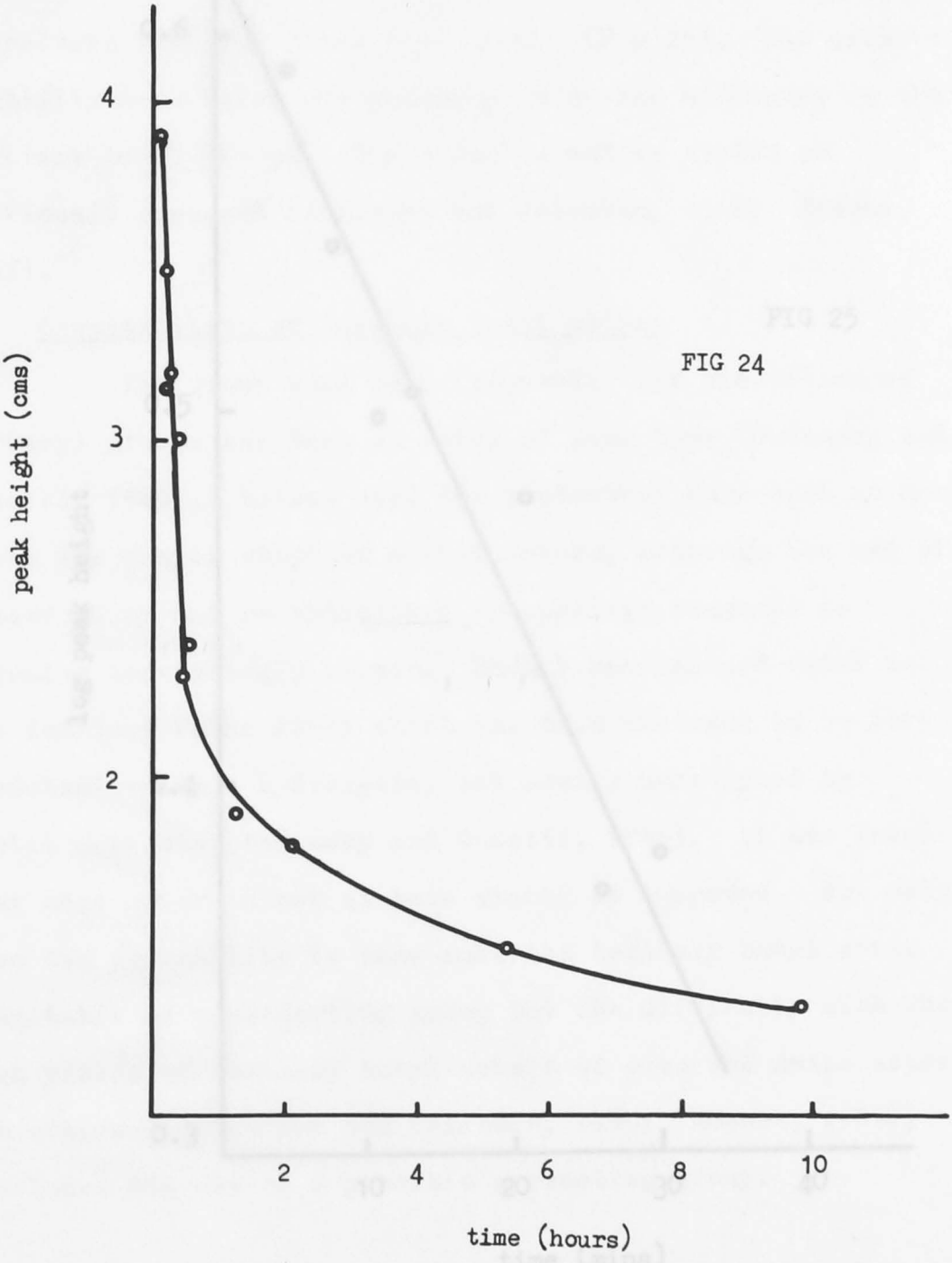


FIG 24

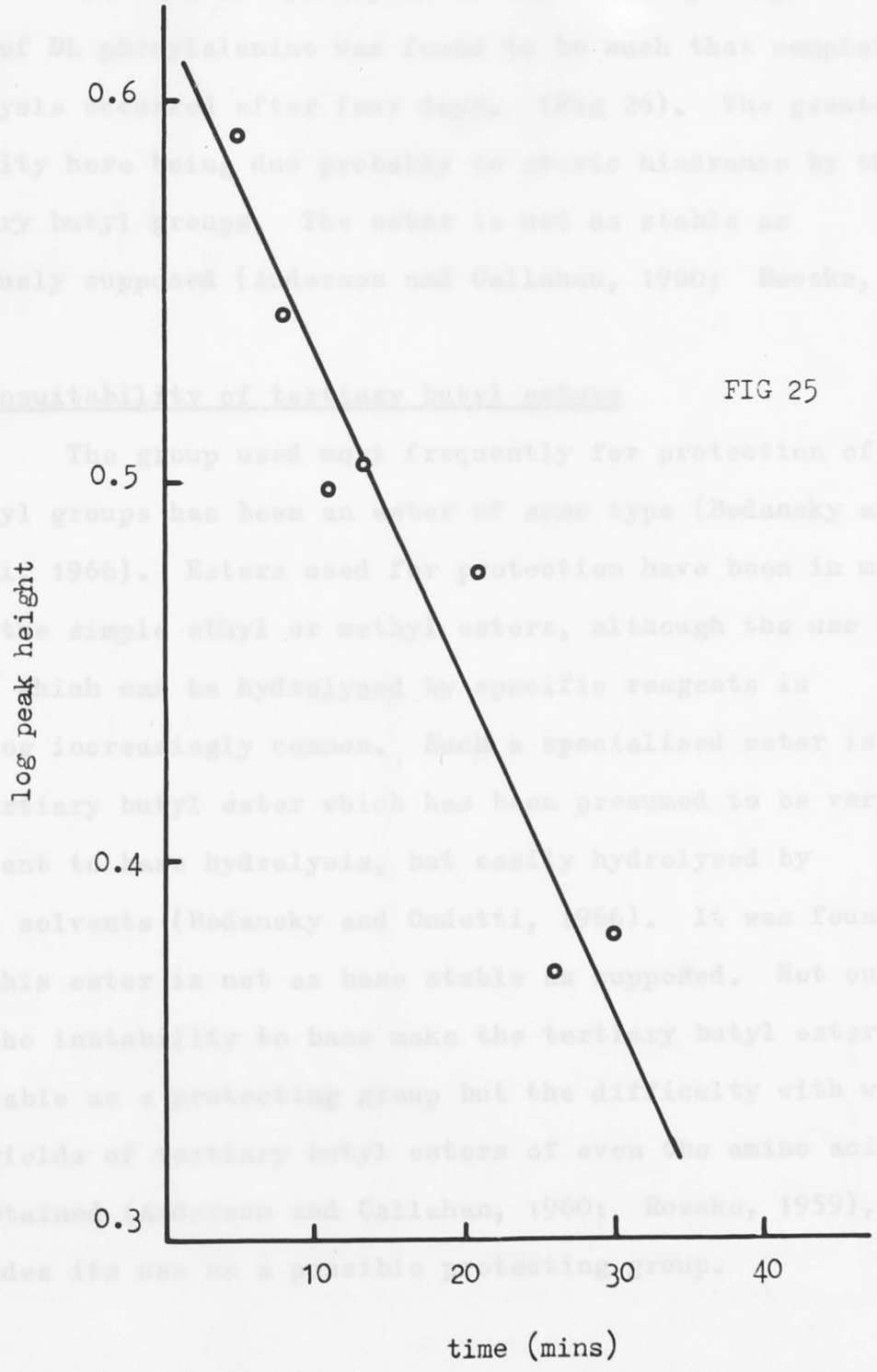


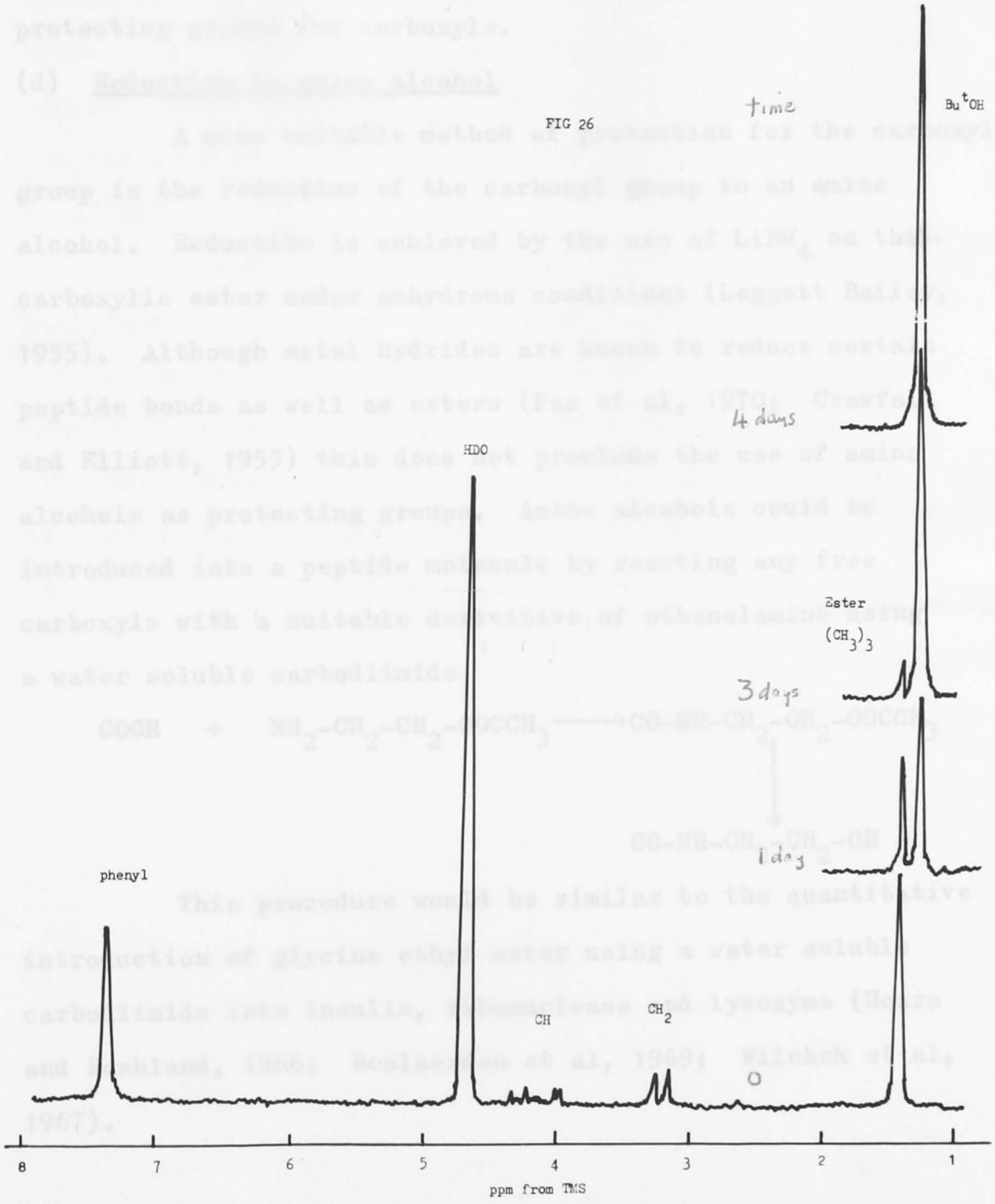
FIG 25

The rate of hydrolysis of the tertiary butyl ester of DL phenylalanine was found to be such that complete hydrolysis occurred after four days. (Fig 26). The greater stability here being due probably to steric hindrance by the tertiary butyl groups. The ester is not as stable as previously supposed (Anderson and Callahan, 1960; Roeske, 1963).

(c) Unsuitability of tertiary butyl esters

The group used most frequently for protection of carboxyl groups has been an ester of some type (Bodansky and Ondetti, 1966). Esters used for protection have been in most cases the simple ethyl or methyl esters, although the use of esters which can be hydrolysed by specific reagents is becoming increasingly common. Such a specialised ester is the tertiary butyl ester which has been presumed to be very resistant to base hydrolysis, but easily hydrolysed by protic solvents (Bodansky and Ondetti, 1966). It was found that this ester is not as base stable as supposed. Not only does the instability to base make the tertiary butyl ester unsuitable as a protecting group but the difficulty with which high yields of tertiary butyl esters of even the amino acids are obtained (Anderson and Callahan, 1960; Roeske, 1959), precludes its use as a possible protecting group.

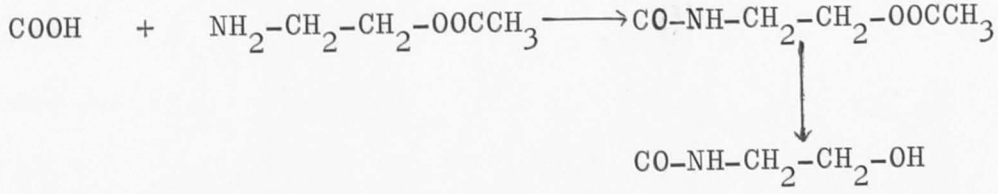
FIG 26


time
Bu^{t}OH
4 days
HDO
Ester
$(CH_3)_3$
3 days
1 day
phenyl
CH
CH_2
O
8
7
6
5
4
3
2
1
ppm from TMS

It is apparent that under the conditions of the sequenation procedure, esters are too unstable to be useful protecting groups for carboxyls.

(d) Reduction to amino alcohol

A more suitable method of protection for the carboxyl group is the reduction of the carboxyl group to an amino alcohol. Reduction is achieved by the use of $LiBH_4$ on the carboxylic ester under anhydrous conditions (Leggett Bailey, 1955). Although metal hydrides are known to reduce certain peptide bonds as well as esters (Paz et al, 1970; Crawfall and Elliott, 1955) this does not preclude the use of amino alcohols as protecting groups. Amino alcohols could be introduced into a peptide molecule by reacting any free carboxyls with a suitable derivitive of ethanolamine using a water soluble carbodiimide



This procedure would be similar to the quantitative introduction of glycine ethyl ester using a water soluble carbodiimide into insulin, ribonuclease and lysozyme (Hoare and Roshland, 1966; Boalaerden et al, 1969; Wilchek et al, 1967).

Accordingly, the ethyl ester of DL alanyl-DL leucyl glycine was reduced to the peptide alcohol with $LiBH_4$ in good yield. Reaction of the peptide alcohol with excess $Co\left[(triene)(OH)(OH_2)\right]^{++}$ resulted in the expected complex containing one cobalt entity per peptide molecule (Fig. 27).

Hydrolysis of the peptide complex at pH 11 and room temperature resulted in the expected $Co\left[(triene)(ala)\right]^{++}$ and DL leucyl-glycinol (Fig 27), the two products being separated by chromatography on carboxy methyl sephedex (Fig 28). However, it was observed that the rate of hydrolysis was not as rapid as previously reported (Buckingham et al, 1970).

FIG 27

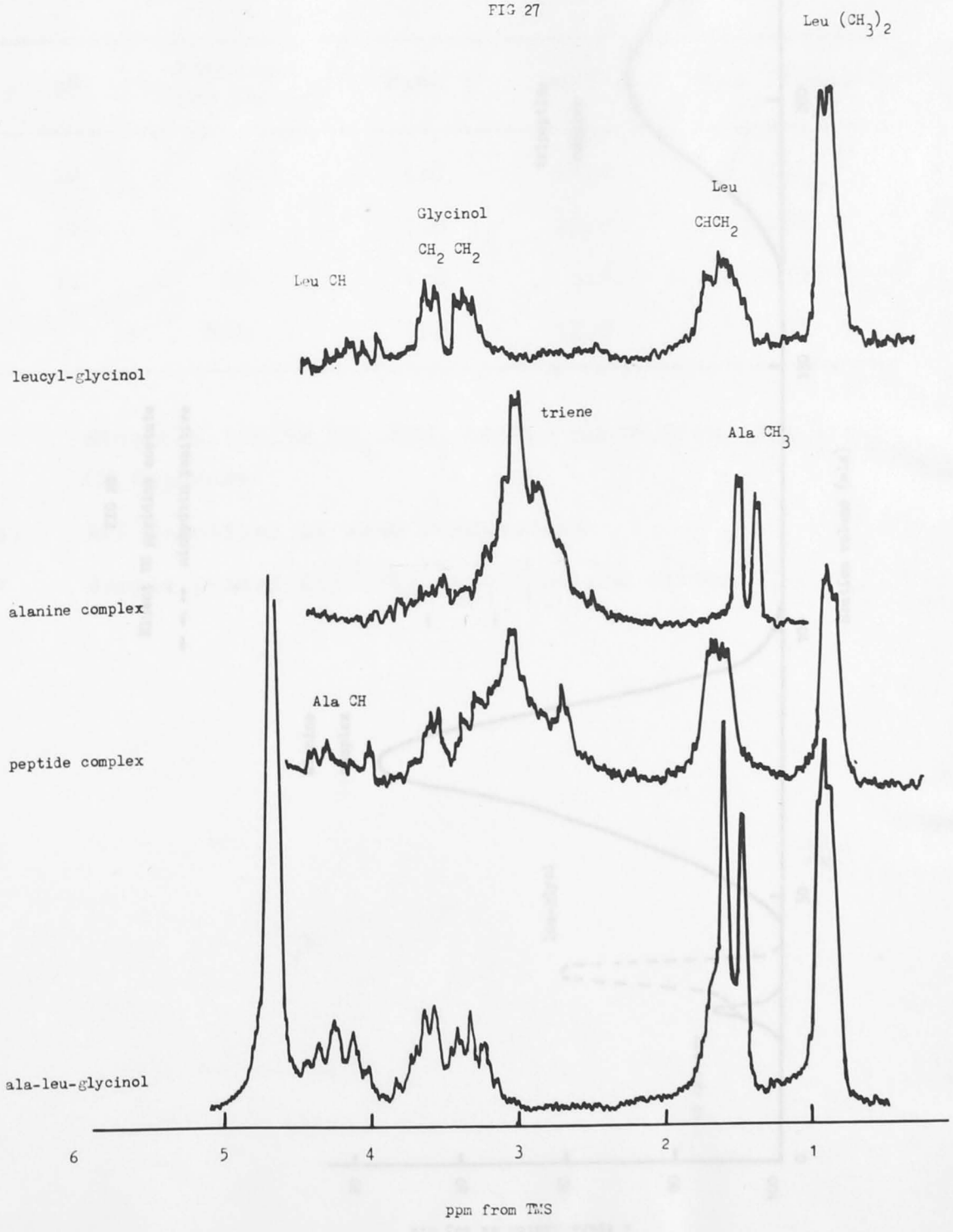

Leu $(CH_3)_2$
Leu
$CHCH_2$
Glycinol
CH_2 CH_2
Leu CH
leucyl-glycinol
triene
Ala CH_3
alanine complex
Ala CH
peptide complex
ala-leu-glycinol
6
5
4
3
2
1
ppm from TMS

FIG 28

Eluent 1M pyridine acetate

– – – ninhydrin positive

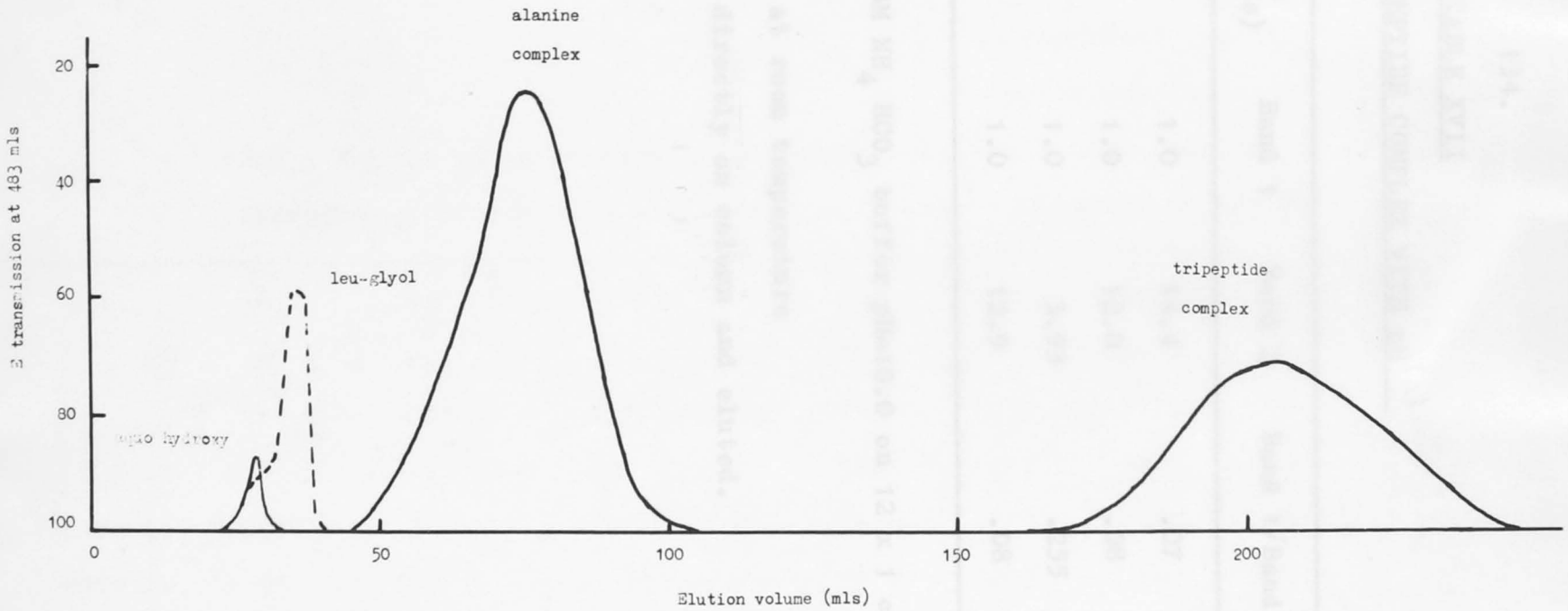


TABLE XVII

STABILITY OF PEPTIDE COMPLEX WITH pH[(a)]

pH	Reaction time (mins)	Band 1	Band 2	Band 1/Band 2
10	60	1.0	14.4	.07
11	60	1.0	12.0	.08
12	60	1.0	3.93	.255
*	NIL	1.0	12.9	.08

Eluent 0.1-0.3M NH_4 HCO_3 buffer pH=10.0 on 12 x 1 cm CM Sephadex

(a) All reactions at room temperature

* Sample placed directly on column and eluted.

TABLE XVIII

STABILITY OF PEPTIDE COMPLEX AND VARIATION OF RATE OF HYDROLYSIS WITH TEMPERATURE*

Temp	Band 1	Band 2	Band 3	Band 4	$\frac{\text{Band 1}}{\text{Band 2}}$	$\frac{\text{Band 4}}{\text{Band 1+2+3}}$	$\frac{\text{Band 3}}{\text{Band 2}}$	$+\frac{1}{2}$
(a)	(b)	5.09	2.85	1.0	(b)	(b)	.56	42 mins
35	0.6	7.21	0.45	1.0	.08	.12	.062	15 mins
35(c)	0.72	8.8	Nil	1.0	.08	.11	-	
40	1.31	8.54	Nil	1.0	.15	.10	-	
45	1.45	5.53	Nil	1.0	.26	.14	-	
45(d)	1.54	9.10	Nil	1.0	.16	.11	-	

Eluent 1M pyridine acetate pH=5.2 on 12 x 1 cm column CM Sephadex

* Except where otherwise stated all reactions at pH 11 for 1 hour

(a) Room temperature

(b) Not determined

(c) Reaction time 2 hours

(d) pH=10.5

Band 1 $Co\left[(triene)(OH)(OH_2)\right]^{++}$

Band 2 $Co\left[(triene)(ala)\right]^{++}$

Band 3 $Co\left[(triene)(ala\text{-}leu\text{-}glycinol)\right]^{+++}$

Band 4 Unknown

HYDROLYSIS OF THE COBALT COMPLEX OF ALANYL-LEUCYL-GLYCINOL

The rate of hydrolysis was determined in the following way. The pH of a known volume of a stock solution of the peptide complex was raised to the desired pH by the addition of 1M NaOH. After a predetermined time the reaction was quenched by lowering the pH to less than 5 with 1M acetic acid. The mixture was then lyophilised to dryness, dissolved in a small amount of eluent (1-2 mls) and chromatographed on carboxy methyl sephadex using either 1M pyridine acetate (pH 5.2) or a 0.1-0.3M ammonium bicarbonate (pH=10.0) as eluent (Figs 16, 29). The various coloured bands were collected on elution, lyophilised to dryness, made up to a standard volume and the optical density at 483 mμ determined. The ratios of the optical densities for the various bands separated from the experiments conducted are given in Tables XVII, XVIII.

Bands 1, 2 and 3 were identified by nmr spectroscopy as $Co\left[(triene)(OH)(OH_2)\right]^{++}$, $Co\left[(triene)(ala)\right]^{++}$ and $Co\left[(triene)(ala\text{-}leu\text{-}glycinol)\right]^{+++}$ respectively. Band 4 could not be identified from its nmr spectrum and is probably due to an impurity in the peptide sample (Fig 30).

FIG 29

Eluent 0.1 - 0.3 M ammonium

bicarbonate gradient

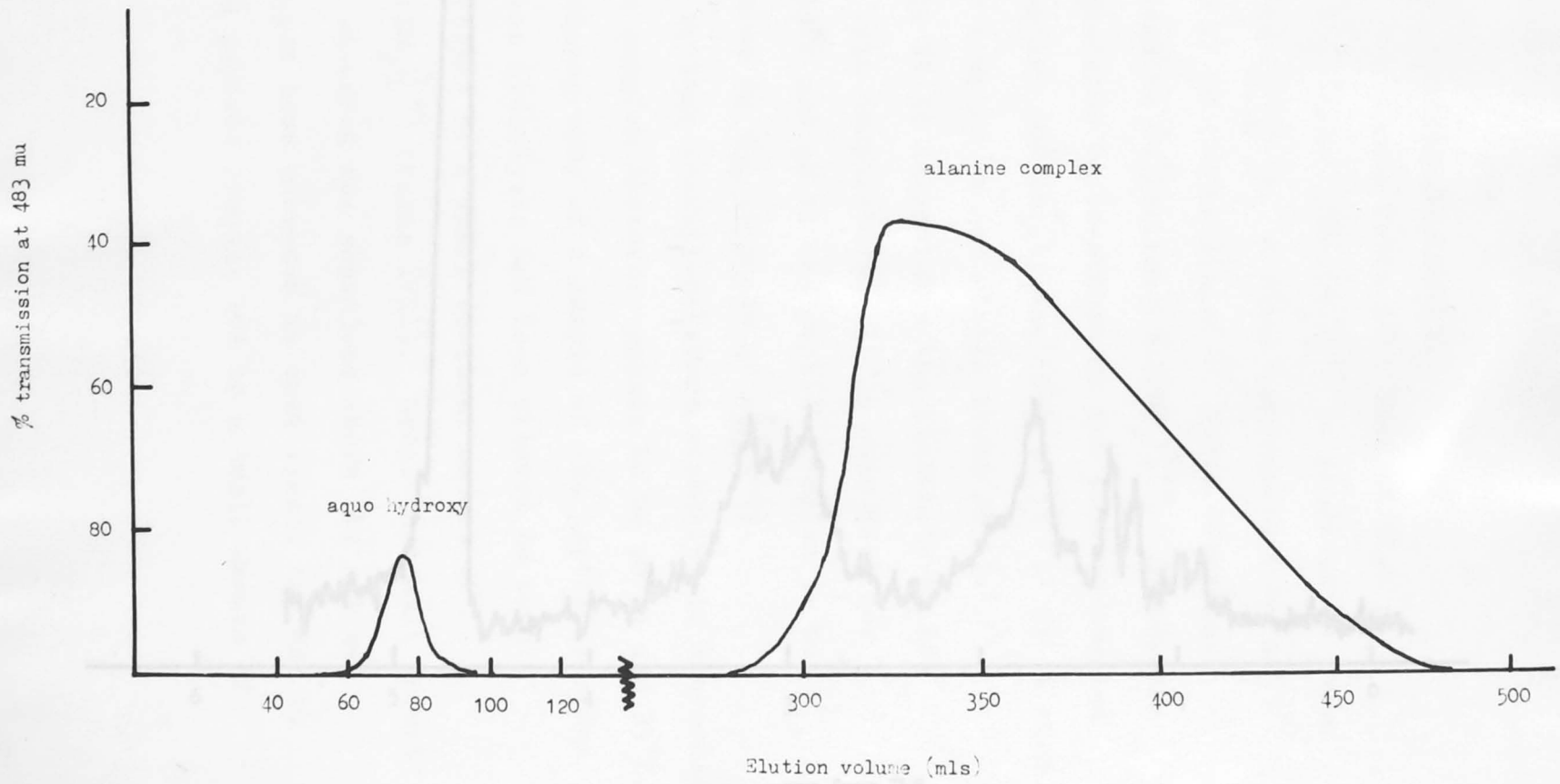
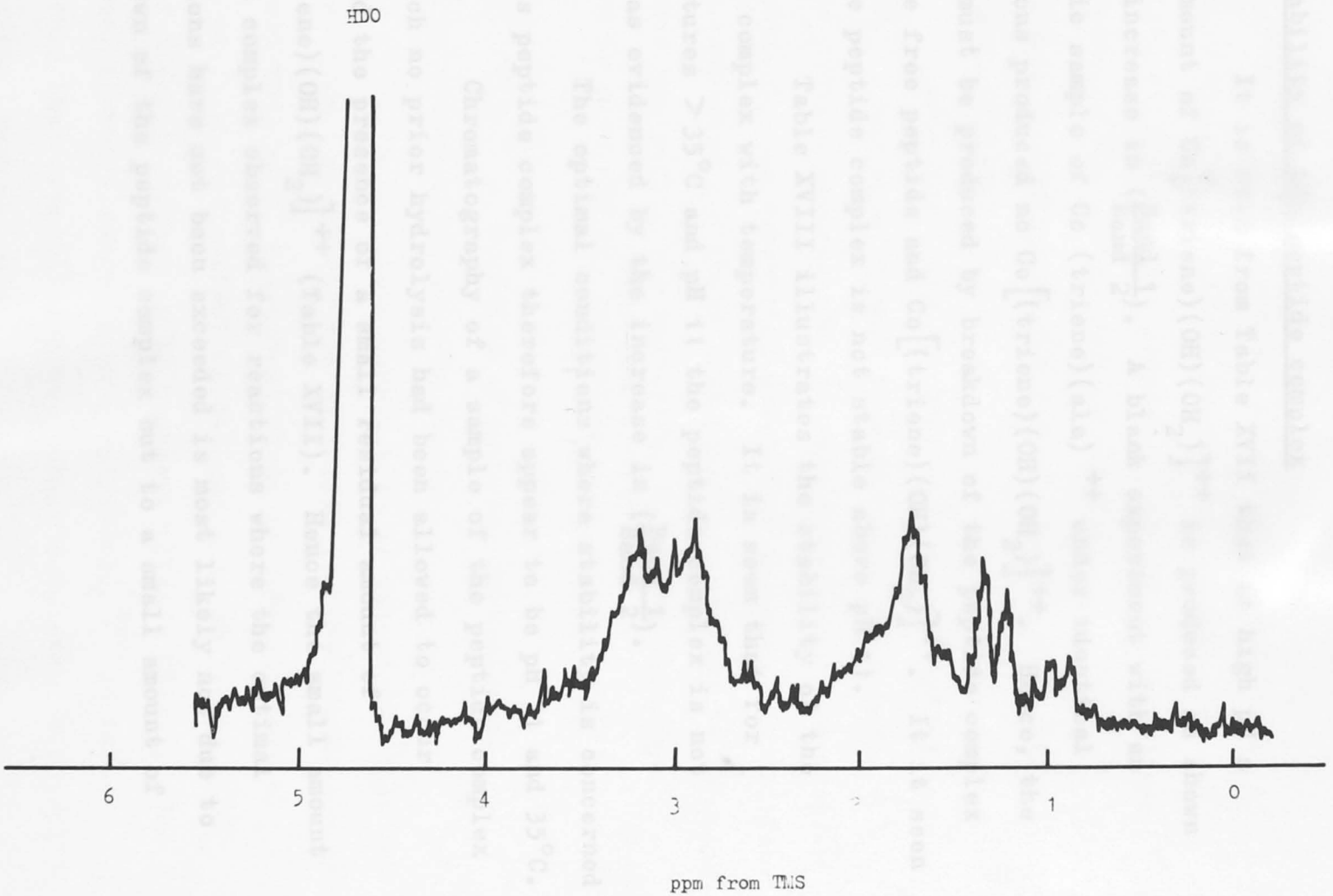


FIG 30



(a) Stability of the peptide complex

It is seen from Table XVII that at high pH a large amount of $Co\left[(triene)(OH)(OH_2)\right]^{++}$ is produced as shown by the increase in $(\frac{Band\ 1}{Band\ 2})$. A blank experiment with an authentic sample of Co (triene)(ala) $^{++}$ under identical conditions produced no $Co\left[(triene)(OH)(OH_2)\right]^{++}$. Hence, the excess must be produced by breakdown of the peptide complex into the free peptide and $Co\left[(triene)(OH)(OH_2)\right]^{++}$. It it seen that the peptide complex is not stable above pH 11.

Table XVIII illustrates the stability of the peptide complex with temperature. It is seen that for temperatures $> 35^{o}C$ and pH 11 the peptide complex is not stable as evidenced by the increase in $(\frac{Band\ 1}{Band\ 2})$.

The optimal conditions where stability is concerned for this peptide complex therefore appear to be pH 11 and $35^{o}C$.

Chromatography of a sample of the peptide complex for which no prior hydrolysis had been allowed to occur revealed the presence of a small residual amount of $Co\left[(triene)(OH)(OH_2)\right]^{++}$ (Table XVII). Hence the small amount of this complex observed for reactions where the optimal conditions have not been exceeded is most likely not due to breakdown of the peptide complex but to a small amount of

$Co\left[(triene)(OH)(OH_2)\right]^{++}$ present in the stock solution. It is thought that this residual complex is probably due to incomplete chromatographic separation of the two complexes during preparation of the peptide complex.

(b) Rate of hydrolysis of the cobalt complex of alanyl-leucyl-glycinol

In calculating the half-life of the hydrolysis reaction, it has been assumed that the extinction coef. of both the peptide complex and the resultant amino acid complex are identical, an assumption which from measurements of other peptide complexes and amino acid complexes appears to be valid. Under this assumption the half-life, t, of the hydrolysis reaction is given by the following:-

$$(\tfrac{1}{2})^{\frac{n}{t}} = \text{fraction of peptide complex remaining}$$

Where n is the reaction time in minutes.

Using the above equation it is calculated that for pH 11 and room temperature the half life for the hydrolysis of the cobalt complex of ala-leu-glycinol is approximately 42 minutes while at the optimal conditions i.e. pH 11 and 35°C the half life is reduced to 15 minutes. (Table XVIII). The rate found for $Co\left[(triene)(ala\text{-}leu\text{-}glycinol)\right]^{+++}$ is much slower than that reported for the cobalt complexes of glycyl peptides (Buckingham, 1970).

RATE OF REACTION OF Co (triene)(OH)$(OH_2)^{++}$

WITH C-TERMINAL PROTECTED PEPTIDES

The rate of reaction was followed by the change in optical densities at 483 mμ and 505 mμ . These wavelengths were chosen as they correspond to the position of maximum absorption for the peptide complex and Co (triene)(OH)$(OH_2)^{++}$ respectively.

The optical densities at the two different wavelengths are given by the following:-

$$D_{505} = E_1C_1 + E_2^{max} C_2 \qquad (A)$$

$$D_{483} = E_1^{max} C_1 + E_2C_2 \qquad (B)$$

where E_2 and E_2^{max} are the extinction coefficients of the aquohydroxy complex at 483 and 505 mμ respectively. E_1^{max} and E_1 are the extinction coefficients of the peptide complex at 483 and 505 mμ respectively. C_1 and C_2 are the concentrations of the peptide complex and aquohydroxy complex respectively.

From equations (A) and (B)

$$C_1 = (E_2D_{505} - E_2^{max} D_{483})(E_1E_2 - E_1^{max} E_2^{max})^{-1}$$

$$= (E_2D_{505} - E_2^{max}D_{483})(K')^{-1}$$

For a first order reaction:-

$$\log a^o/a = kt/2.3$$

$$\text{i.e.} \quad -\log a = kt/2.3 - \log a^o$$

when a^o = initial concentration of peptide complex

a = concentration at time t

Thus $\log (K')/(E_2D_{505} - E_2^{max} D_{483}) = kt/2.3 - \log a^o$

$\therefore \log (E_2 D_{505} - E_2^{max} D_{483}) = kt/2.3 + \log a^o + \log K'$

$= \log M$

Hence for a first order reaction a plot of $\log (E_2D_{505} - E_2^{max} D_{483})$ against t should be linear with slope - k/2.3.

For a second order reaction where the initial concentrations are unequal:-

$$kt = (b-a)^{-1} \ln a(b-x)/b(a-x)$$

where a and b are the initial concentrations of the two reactants and x is the concentration reacted at time t. Hence for a second order reaction a plot of $\log a(b-x)/b(a-x)$ against t will be linear with a slope of $k(b-a)/2.3$.

In the case where the initial concentrations of the reactants are equal the rate equation is given by:-

$$(a^o-a)/a^o a = kt$$

where the designations are the same as before.

Thus $(a)^{-1} = kt + (a^o)^{-1}$

Hence $(K')/(E_2D_{505} - E_2^{max} D_{483}) = kt + (a^o)^{-1}$

$$(E_2D_{505} - E_2^{max} D_{483})^{-1} = kt/K' + (a^oK')^{-1}$$

$$= E^{-1}$$

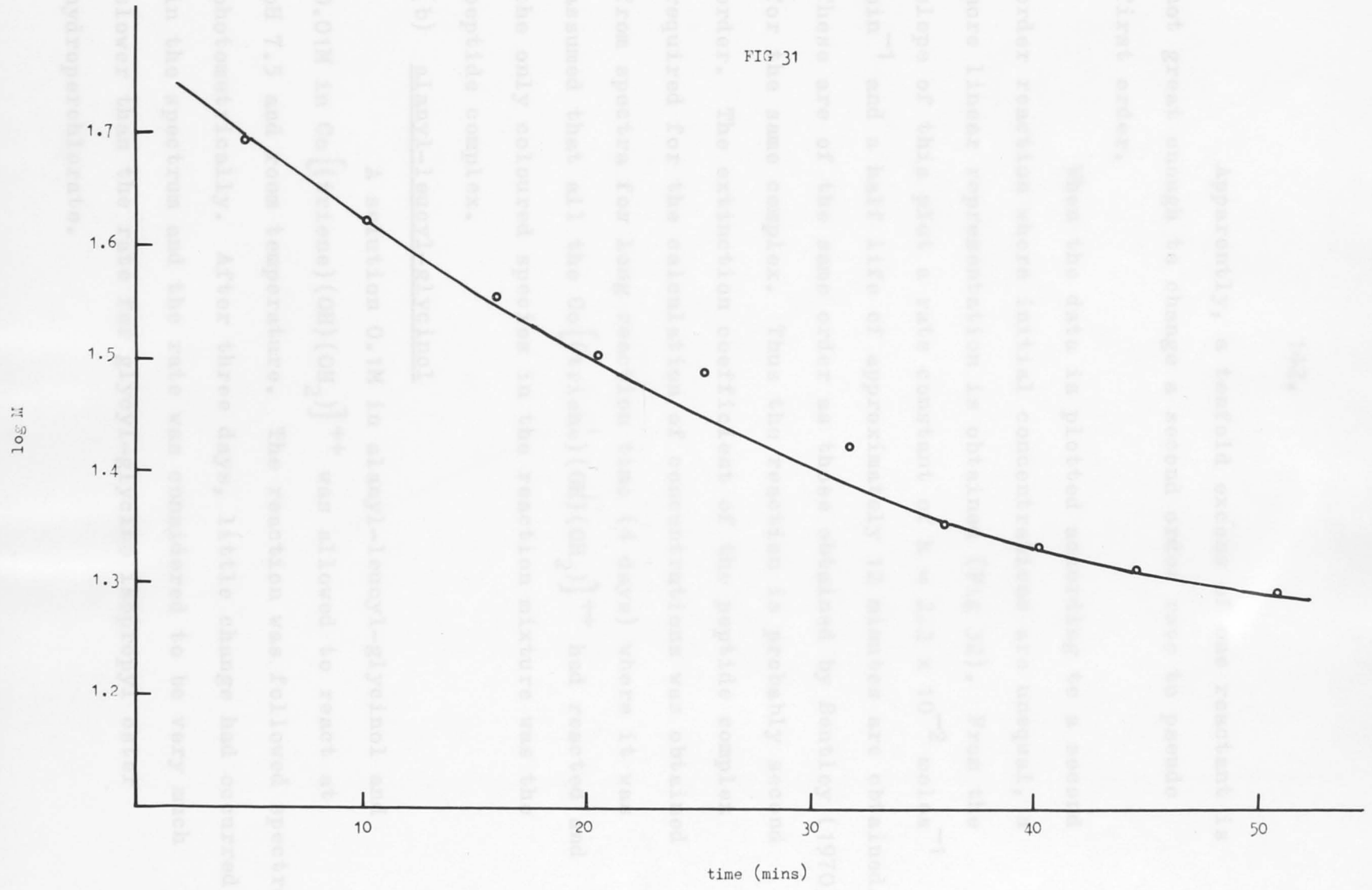
Thus a plot of $(E_2D_{505} - E_2^{max} D_{483})^{-1}$ against t will be linear with slope k/K'. K' is obtained from the intercept.

(a) Glycyl-glycine isopropyl ester hydroperchlorate

A solution 0.1M in glycyl-glycine isopropyl ester hydroperchlorate and 0.01M in $Co\left[(triene)(OH)(OH_2)\right]^{++}$ was allowed to react at pH 7.5 and room temperature. Spectra were obtained between 700 mμ and 300 mμ at a high scanning speed (50 A^o per minute) and at approximately five minute intervals. The time of the spectrum was taken at the moment the scan passed 500 mμ. No buffer was added to the reaction mixture. However, no change was found in pH before and after the reaction.

With the concentrations used one would expect the pseudo reaction to be first order. However, a plot of log $(E_2D_{505} - E_2^{max} D_{483})$ against time appears to be non-linear (Fig 31). Nevertheless, a linear interpretation of the plotted data leads to a half life of approximately 30 minutes for the reaction.

FIG 31



Apparently, a tenfold excess of one reactant is not great enough to change a second order rate to pseudo first order.

When the data is plotted according to a second order reaction where initial concentrations are unequal, a more linear representation is obtained (Fig 32). From the slope of this plot a rate constant of $k = 2.2 \times 10^{-2}$ moles^{-1} min^{-1} and a half life of approximately 12 minutes are obtained. These are of the same order as those obtained by Bentley (1970) for the same complex. Thus the reaction is probably second order. The extinction coefficient of the peptide complex required for the calculation of concentrations was obtained from spectra for long reaction time (4 days) where it was assumed that all the $Co\left[(triene)(OH)(OH_2)\right]^{++}$ had reacted and the only coloured species in the reaction mixture was the peptide complex.

(b) <u>alanyl-leucyl-glycinol</u>

A solution 0.1M in alanyl-leucyl-glycinol and 0.01M in $Co\left[(triene)(OH)(OH_2)\right]^{++}$ was allowed to react at pH 7.5 and room temperature. The reaction was followed spectro photometrically. After three days, little change had occurred in the spectrum and the rate was considered to be very much slower than the rate for glycyl-glycine isopropyl ester hydroperchlorate.

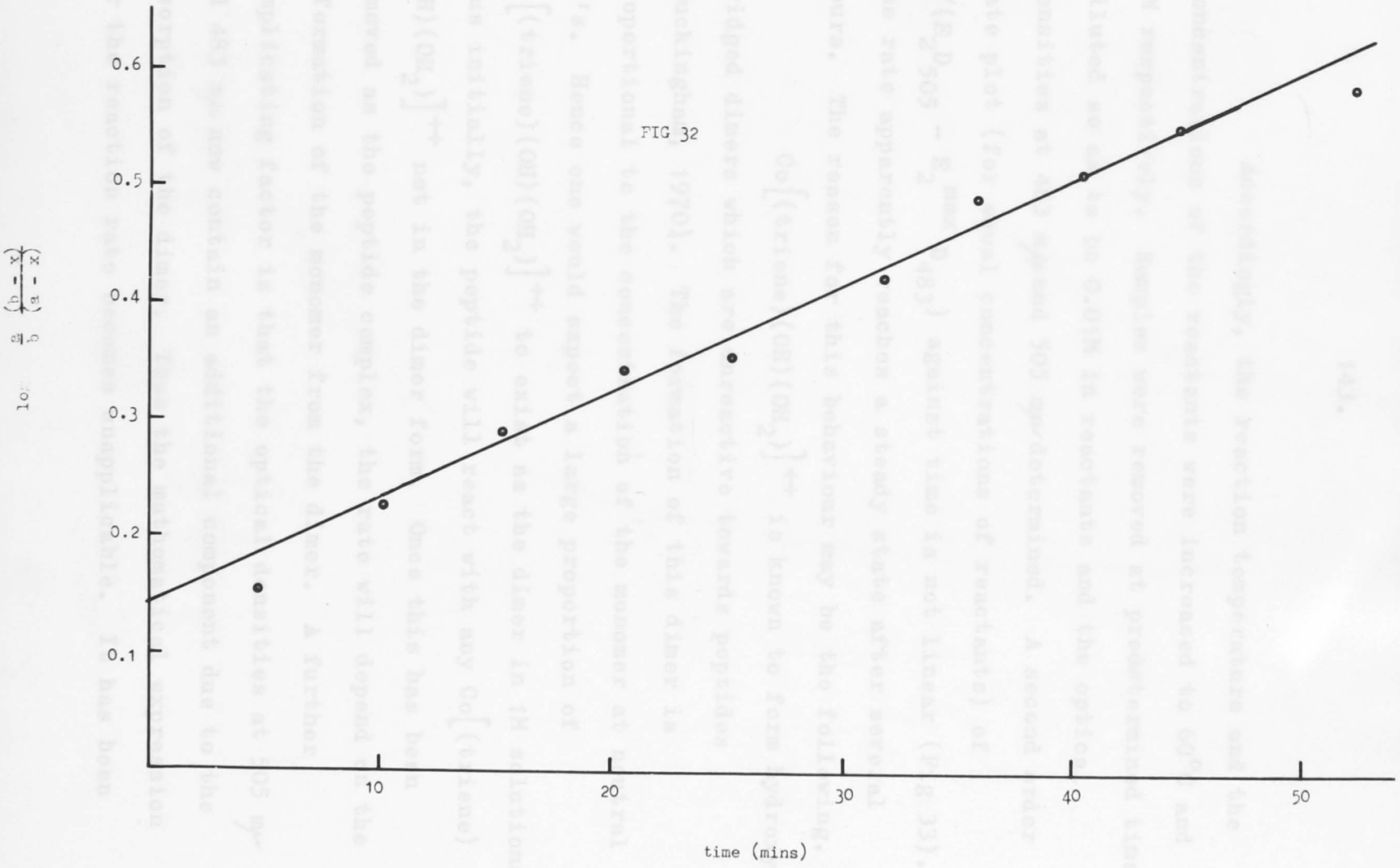
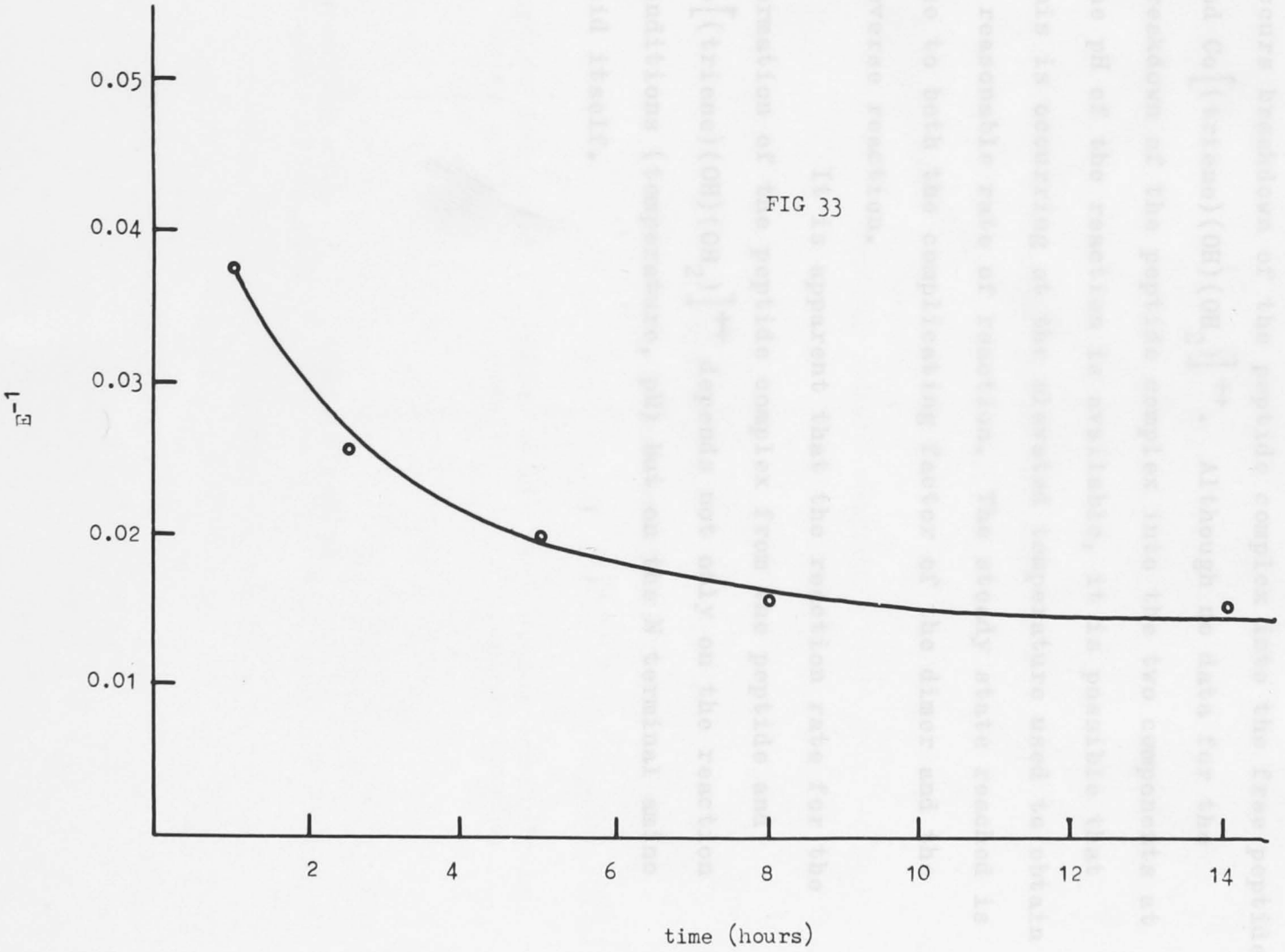


FIG 32
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
time (mins)

Accordingly, the reaction temperature and the concentrations of the reactants were increased to 60°C and 1M respectively. Samples were removed at predetermined times diluted so as to be 0.01M in reactants and the optical densities at 483 mμ and 505 mμ determined. A second order rate plot (for equal concentrations of reactants) of $1/(E_2D_{505} - E_2^{max} D_{483})$ against time is not linear (Fig 33). The rate apparently reaches a steady state after several hours. The reason for this behaviour may be the following.

$Co\left[(triene)(OH)(OH_2)\right]^{++}$ is known to form hydroxy bridged dimers which are unreactive towards peptides (Buckingham, 1970). The formation of this dimer is proportional to the concentration of the monomer at neutral pH's. Hence one would expect a large proportion of $Co\left[(triene)(OH)(OH_2)\right]^{++}$ to exist as the dimer in 1M solutions. Thus initially, the peptide will react with any $Co\left[(triene)(OH)(OH_2)\right]^{++}$ not in the dimer form. Once this has been removed as the peptide complex, the rate will depend on the reformation of the monomer from the dimer. A further complicating factor is that the optical densities at 505 mμ and 483 mμ now contain an additional component due to the absorption of the dimer. Thus the mathematical expression for the reaction rate becomes unapplicable. It has been

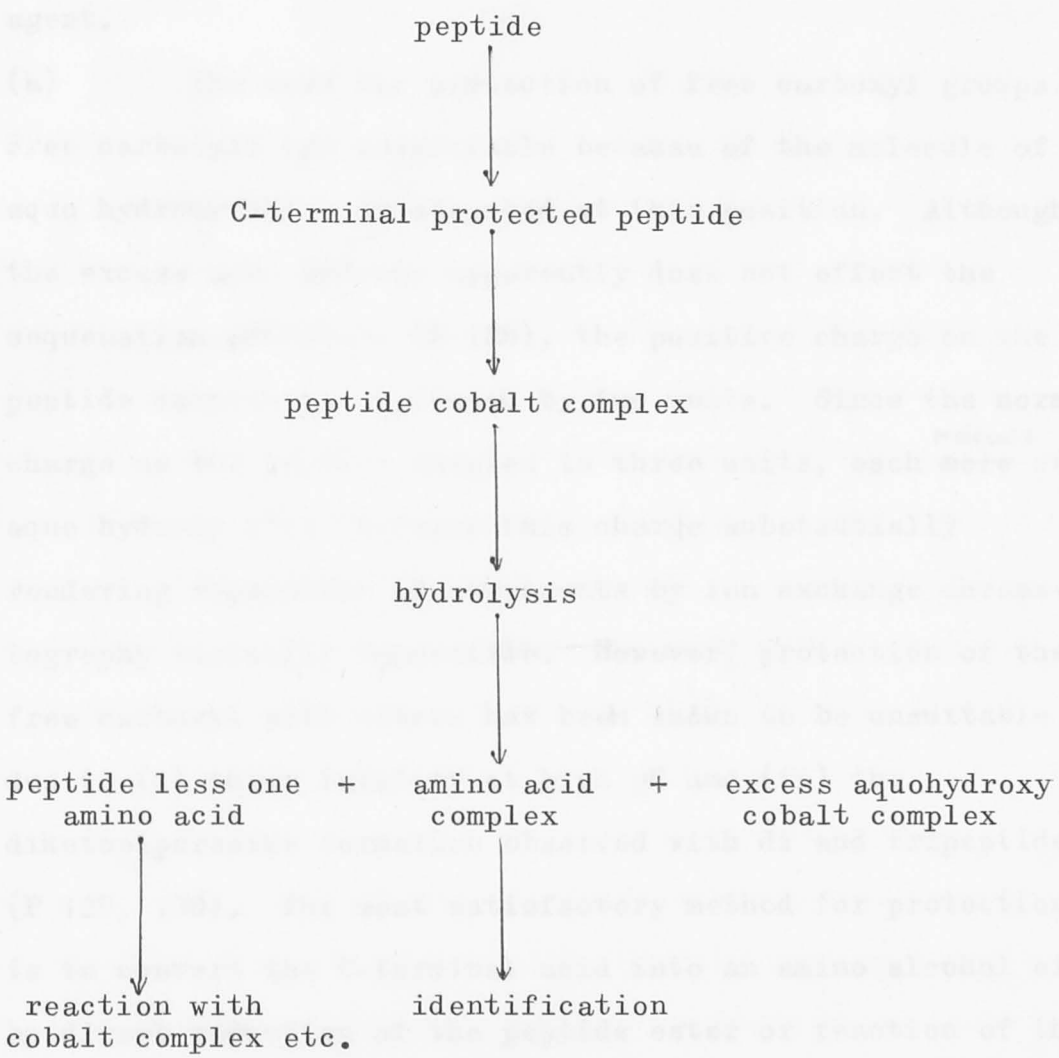
FIG 33



observed that at elevated temperatures and high pH there occurs breakdown of the peptide complex into the free peptide and $Co\left[(triene)(OH)(OH_2)\right]^{++}$. Although no data for the breakdown of the peptide complex into the two components at the pH of the reaction is available, it is possible that this is occurring at the elevated temperature used to obtain a reasonable rate of reaction. The steady state reached is due to both the complicating factor of the dimer and the reverse reaction.

It is apparent that the reaction rate for the formation of the peptide complex from the peptide and $Co\left[(triene)(OH)(OH_2)\right]^{++}$ depends not only on the reaction conditions (temperature, pH) but on the N terminal amino acid itself.

CONCLUSION


peptide
C-terminal protected peptide
peptide cobalt complex
hydrolysis
peptide less one amino acid
+
amino acid complex
+
excess aquohydroxy cobalt complex
reaction with cobalt complex etc.
identification

There appears to be several problems associated with the use of $Co\left[(triene)(OH)(OH_2)\right]^{++}$ as a sequenating agent.

(a) The need for protection of free carboxyl groups. Free carboxyls are undesirable because of the molecule of aquo hydroxy which is absorbed at this position. Although, the excess aquo hydroxy apparently does not effect the sequenation procedure (P 128), the positive charge on the peptide complex is increased by two units. Since the normal charge on the peptide complex is three units, each ~~mole~~ molecule of aquo hydroxy will increase this charge substantially rendering separation of components by ion exchange chromatography virtually impossible. However, protection of the free carboxyl with esters has been shown to be unsuitable due to (i) their lability at high pH and (ii) the diketopiperazine formation observed with di and tripeptides (P 129, 130). The most satisfactory method for protection is to convert the C-terminal acid into an amino alcohol either by direct reduction of the peptide ester or reaction of the carboxyl group with ethanolamine (P 132).

(b) Rate of formation of the peptide complex

The rate of formation of the peptide complex of glycyl-glycine isopropyl ester was observed to be a great deal faster than the rate of formation of the complex of DL alanyl-DL leucyl-glycinol (P 139). It is apparent that this rate depends to a great extent on the nature of the N terminal residue. Not only this, but in all probability, the rate of reaction of longer peptides could be slower still. This would occur undoubtedly if secondary structures in the peptide, promoted by intramolecular hydrogen bonding, 'buried' the N terminal residue as occurs in many proteins.

One method of increasing the rate is, naturally, to increase the concentration of the reactants. This is, unfortunately, precluded by the fact that at high concentrations formation of the unreactive dimer is encouraged (P 143)*. The slow rate of formation of the peptide complex must be considered as a serious disadvantage. Additional experimental data is required on the rate of formation of complexes of peptides of a larger length and with N terminal residues other than glycine and alanine, which are of course the least sterically hindered of amino acids. It may be possible also to find a catalyst for the reaction.

* Note added in proof.
Recently, Bently (personal communication) has found that formation of the dimer is retarded by the use of aqueous acetone as a solvent.

(c) Hydrolysis of the peptide complex

The experimental data obtained for the rate of hydrolysis of the complex of DL alanyl-DL leucyl-glycinol again indicates the dependence of the rate of reaction on the N terminal residue (P 138). The rate found was much slower than that observed for glycine peptide complexes. Furthermore, any attempt to increase this rate by increasing the temperature or pH resulted in breakdown of the peptide complex into the free peptide and the aquo hydroxy complex (P 137). This instability must be considered as a serious disadvantage. The non-quantitative removal of amino acids from the peptide chain would seriously limit the extent to which the method could proceed along the chain. A stage will be reached where large numbers of different amino acid complexes, originating from chains where all amino acid residues have been incompletely removed, are released at each step. Such will make correct determination of the amino acid released impossible. Thus one must keep temperatures and pH to a minimum which of course decreases the rate of hydrolysis. Again more data is required for peptides containing N terminal residues other than alanine and glycine and as with (b) it may be possible to catalytically increase the rate of hydrolysis.

(d) Separation of reaction products

Ideally, separation of reaction products could be best achieved by the use of a solid support for the peptide. However, this did not prove successful due to the catalytic effect of the resin on the peptide, the peptide complex and the amino acid complex released (P 119). It is felt that further developmental work with support polymers could satisfactorily overcome this problem (P 126), and additional experimentation may be fruitful. Even so, the method used for separation of reaction products leaves much to be desired and a more suitable method would be where either the peptide complex and amino acid complex or the residual peptide could be extracted in a non-miscible solvent. As yet no such solvent is available. Even so, the separation procedure cannot be considered as a serious disadvantage.

(e) Identification of amino acid complex

Identification of the amino acid complex appears to offer no serious difficulties (P 113). Although nmr spectra are considered to be the least ambiguous of the methods investigated, chromatography appears to be the most suitable requiring much less sample.

It can be concluded that the method cannot, in its present state, be considered as a serious competitor to the already well established methods of sequenation. Nevertheless, in the event of the conditions discussed above being satisfied, the method must be considered as an alternative to other methods. Even so, the method may find use, even with the difficulties discussed, where a particularly mild method of sequenation or N terminal determination is required.

APPENDIX A

Yu and Stockmayer (1967) calculated that the viscosity of two long rods of equal length connected by a flexible joint was related to the fully rigid rod by the following equation:-

$$[\eta']/[\eta] = 1-0.60(1-f)f$$

where $[\eta']$ is the viscosity of the once broken rod

$[\eta]$ is the viscosity of the rigid rod

f = molar length of broken section

i.e. f = (n-m)/n where n = no. of turns

m = position of break

Assuming one break with no restriction on the position of the break. Then averaging over all molecules for all positions.

$$= \frac{1}{n} \sum_{1}^{n} (1-0.6f(1-f))$$

$$= \frac{1}{n} \sum 1 - 6/10n^3 \sum (n-m)(n)$$

$$= 1 - 3(n+1)/10n + (n+1)(2n+1)/10n^2$$

$$= 1 + (1-n^2)/10n^2$$

$$= 0.9 + 1/10n^2$$

Hence the viscosity drop for one break with no restriction on the position of the break is approximately 10%.

APPENDIX B

Calculation of number of monomer units at surface of resin beads

Assume 1. resin beads spherical

2. monomer unit occupies a spherical volume

3. each resin bead contains n monomer spheres of which m monomer units are at the surface.

True Volume of resin bead $= (4/3)\pi R^3$ R = radius of bead

Apparent volume of resin bead $= n((4/3)\pi R'^3)$ R' = radius of monomer unit

The true volume is not equal to the apparent volume due to interstitial volume. However, an estimate of this volume may be obtained in the following way.

If it is assumed that n^3 spheres are packed into a cube

then the side of the cube = 2nR

where R is the radius of the cube

Hence the true volume of the cube $= (2nR)^3 = V_t$

and the apparent volume $= n^3((4/3)\pi R^3) = V_a$

$\therefore$ packing error, $k = V_t/V_a = \pi/6$

Hence true volume of resin = $\pi/6$ apparent volume of resin

$$(4/3)\ R^3 = ((4/3)\ R'^3)\ \pi/6$$

$$\therefore \quad = R^3 / R'^3\ 6/\pi$$

Assuming that the surface of the resin bead is covered with hemispheres of monomer units.

Surface area of bead $= 4\pi R^2$

$m(0.5)4\pi(R')^2$ for $R \gg R'$

$\therefore \quad m = 2\,R^2/R'^2$

Hence $\quad m/n = (R'/R)\ \pi/3$

The radius of the monomer unit estimated from models of p-amino ethyl phenyl ethylene was $5A^o$.

Radius of beads

50 mesh $\quad$ Radius $= (0.5)(2.5)/50$ cms

$= 2.5 \times 10^6\ A^o$

200 mesh $\quad$ Radius $= (0.5)(2.5)/200$ cms

$= 6.25 \times 10^5\ A^o$

50 mesh $\quad m/n = (5/2.5 \times 10^6)\ /3$

$= 2 \times 10^{-6}$

200 mesh $\quad m/n = (5/6.25 \times 10^5)\ /3$

$= 8 \times 10^{-6}$

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HELIX TO COIL TRANSITION IN POLY-L-AMINO ACIDS

II.* N.M.R. STUDY OF MODEL COMPOUNDS AND POLY-γ-BENZYL-L-GLUTAMATE

By J. H. Bradbury† and M. D. Fenn†

[Manuscript received September 27, 1968]

Summary

The proton magnetic resonance spectroscopy of simple amides and poly-L-amino acids has been investigated with particular reference to the question of charging of the amide group in strong acids. It is found that the downfield chemical shift of the α-CH resonance of poly-γ-benzyl-L-glutamate (PBG) which accompanies the helix to coil transition can be conveniently divided into two parts on the basis of experiments with model systems. The first is due to the collapse of the helix to an uncharged random coil and the second represents the charging of the amide groups of the random coil.

For PBG samples of low molecular weight, two α-CH resonances are observed due to residues in helical (uncharged) and random-coil (charged) forms. The rate of exchange between them is of the order of 400 sec^{-1}. The proton resonances of the NH proton, COOH proton of the acid, and α-CH proton of dichloroacetic acid (DCA) are shown to be less useful than the α-CH proton.

A critical evaluation has been made of all the evidence relevant to the charging of the amide group of polypeptides in mixtures of non-interacting solvents and organic acids e.g. trifluoroacetic acid (TFA) and DCA. It is concluded that viscosity, infrared, dielectric constant, electric birefringence, and conductivity studies give overwhelming support to the concept of charging. Nuclear magnetic resonance, circular dichroism, and optical rotatory dispersion are less sensitive indicators of small amounts of charging, and hence studies should be made with samples of low molecular weight (where there is a greater fractional degree of charging). Such studies made here by n.m.r. methods give strong support for charging.

The features of the flexible helix model first proposed in Part I are delineated. It consists of helical segments separated by short, charged random-coil breaks at both ends and in the interior of the molecule. Although elaborated in some detail for the case of PBG, it is likely to occur quite generally for those polypeptides which are soluble in organic solvents and undergo the helix to coil transition.

Introduction

It has been demonstrated clearly by viscometry,[1,2] in confirmation of earlier work using other techniques,[3–5] that PBG and other poly-L-amino acids are charged in DCA and TFA, and in mixtures of these with non-interacting solvents. Since nuclear magnetic resonance spectroscopic studies had proved useful in the observation of charging effects in proteins[6] it was decided to use this technique on PBG

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solutions. At that time, n.m.r. studies had been made on PBG[7] and on poly-γ-ethyl-L-glutamate.[8] During the course of this work,[1] evidence has accumulated from n.m.r.,[9,10] circular dichroism (c.d.),[11] and optical rotatory dispersion (o.r.d.)[12] studies which purports to show the absence of charging of the peptide bond of poly-L-amino acids in strong acid solvents. It is the main object of this paper to examine this question in some detail by n.m.r. techniques.

A subsidiary problem, which arises in the interpretation of the chemical-shift data of the α-CH proton resonances, concerns the exchange between helical and random-coil peptide groups.[9,10,13,14] A complete account of this work will be given subsequently.[15]

Experimental

Materials

The purification of most solvents and the source of the poly-L-amino acids were given in Part I.[2] Additional solvents used were chloroform-*d* and dimethyl sulphoxide-d_6 (Merck), both of spectroscopic grade, piperidine (M. & B.), pyrrolidine (Light), and triethylamine (B.D.H.), of laboratory grade. Analytical reagents used were potassium t-butoxide (M.S.A. Corp.), *N*-isopropylbenzamide, L-lysine (N.B.C.), benzyloxycarbonyl-L-alanine (N.B.C.); laboratory reagents used were n-propylamine (B.D.H.), *N*-ethylaniline (B.D.H.), *N*-methylformamide (B.D.H.), *N*,*N*-dimethylformamide (K. & K.), *N*-methylacetamide (K. & K.), *N*,*N*-dimethylacetamide (K. & K.), diethyl acetaminomalonate (Light), and *N*,*N*-dimethylacrylamide (K. & K.).

The following peptides were kindly supplied by Dr F. H. C. Stewart, Division of Protein Chemistry, CSIRO, Melbourne:[16] Z-Glu(OEt)-Glu(OEt)-OH, Z-Glu(OMe)-Glu(OEt)-OPNB, Z-Glu(OEt)-Glu(OMe)-Glu(OEt)-OPNB, Z-Glu(OMe)-Glu(OMe)-Val-OPNB, Z-[Glu(OMe)-Glu(OEt)]$_2$-Glu(OEt)-Val-OPNB, where Z = benzyloxycarbonyl, PNB = *p*-nitrobenzyl, Val = L-valyl, and Glu(OEt) and Glu(OMe) are γ-ethyl-L-glutamyl and γ-methyl-L-glutamyl respectively.

N.M.R. Methods

Spectra were obtained with a Perkin–Elmer R10 60-MHz spectrometer at 35° using an r.f. input setting of 1 mV. Spectrum accumulation was also available by means of a Digital Equipment PDP8-S computer. Some spectra were obtained using a Varian HA100 spectrometer. All solute concentrations were 10% w/v. In organic solvents, chemical shifts were measured (accuracy ±0·02 p.p.m.) from an internal standard of tetramethylsilane (TMS) and are recorded on the τ scale.[17] Mixed solvents were prepared by mixing accurately known volumes of the two components. In D_2O solutions, pH-meter measurements were made with an accuracy of ±0·04 using a Beckman research pH meter, and pD obtained by the equation pD = pH-meter reading + 0·40.[18,19] Chemical-shift readings were recorded in p.p.m. from the HDO resonance which occurs at essentially constant field over the pD range used here.[20,21]

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Determination of DP_n *by N.M.R.*

It is well known and has recently been confirmed using tracer techniques[22] that in polymerization of *N*-carboxy acid anhydrides with primary amine initiators, the initiator (n-hexylamine) is incorporated at the end of the chain. Thus the polymer has the composition H–[NH–CH–($CH_2CH_2COOCH_2C_6H_5$)–CO]$_n$–$NHCH_2(CH_2)_4CH_3$. From the n.m.r. spectrum in Figure 1 it is possible to obtain the average value of $n = DP_n$, from the ratio of the areas under the phenyl (or benzyl CH_2) resonances and the $(CH_2)_4$ (or CH_3) resonances. The results for three samples are given in Table 1 of Part I and the good agreement between DP_w and DP_n shows that the samples are essentially monodisperse.

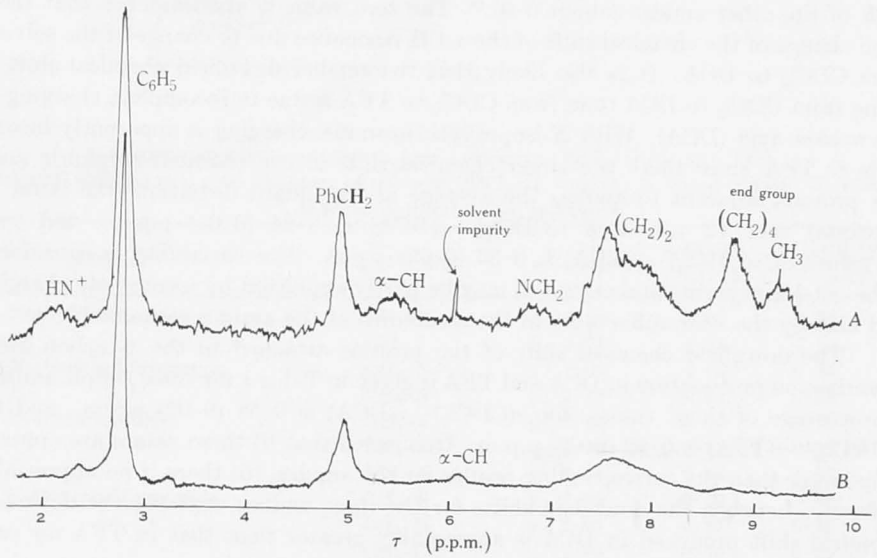
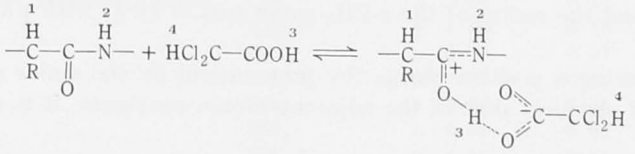


Fig. 1.—N.m.r. spectra of PBG solutions. *A*, sample No. 17 (DP 3·3) in TFA; *B*, sample No. 10 (DP_w 26) in $CDCl_3$.

Results and Discussion

Equation (1) gives the reaction between dichloroacetic acid and a simple amide or peptide group in a polypeptide (in the α-helical form) to produce an ion-pair between the protonated amide or peptide group and dichloroacetate ion. We will consider in turn the n.m.r. spectra of the protons numbered 1–4 in equation (1).



(1)

(a) *Proton 1 (α-CH proton)*

(i) *Amides and Amines*

It is well known that protonation of amides occurs in concentrated aqueous

[22] Terbojevich, M., Pizziolo, G., Peggion, E., Cosani, A., and Scoffone, E., *J. Am. chem. Soc.*, 1967, **89**, 2733.

solutions of strong acids or in strongly protic solvents.[23–27] Protonation of the amide can therefore be effected by transfer of the model amide from $CDCl_3$ to DCA or TFA. Chemical-shift data for this process for various model amides and amines are collected in Table 1. It is noted in columns 4 and 5 that there is a considerable downfield shift of the resonances of protons adjacent to the amide group on charging in DCA or TFA, except in the case of *N*-isopropylbenzamide in DCA where the shift is zero. This is because of the difficulty of charging the amide group of this compound, since its pK_a in aqueous solution ($-2{\cdot}0$) is much lower than that of the bulk of the other amides (about $0{\cdot}0$).[28] The zero value is also evidence that there is no change of the chemical shift of the α-CH resonance due to change of the solvent from $CDCl_3$ to DCA. It is also likely that the smaller downfield chemical shift in going from $CDCl_3$ to DCA than from $CDCl_3$ to TFA is due to incomplete charging in the weaker acid (DCA). With *N*-isopropylbenzamide, charging is apparently incomplete in TFA, since there is a larger chemical shift in concentrated sulphuric acid. For protons adjacent to amides the average and standard deviation (the latter in brackets) over 12 values of $\tau(CDCl_3)-\tau(DCA)$ is $0{\cdot}24$ ($0{\cdot}04$) p.p.m., and over 13 values of $\tau(CDCl_3)-\tau(TFA)$ is $0{\cdot}35$ ($0{\cdot}09$) p.p.m. The variability is considered to be outside experimental error and may be partly explained by incomplete charging and also by the wide differences in the structures of the amides examined.

The downfield chemical shift of the protons attached to the α-carbon atom in amines on protonation in DCA and TFA is given in Table 1 for three simple amines. The average of these values, for $\tau(CDCl_3)-\tau(DCA)$ is $0{\cdot}58$ ($0{\cdot}02$) p.p.m., and for $\tau(CDCl_3)-\tau(TFA)$ is $0{\cdot}52$ ($0{\cdot}02$) p.p.m. It is noted that (i) these values are appreciably larger than the corresponding results for the amides, (ii) there is no appreciable difference between the downfield shifts for the three amines, and (iii) the downfield chemical shift produced in DCA is appreciably greater than that in TFA for each amine.

The chemical-shift data of L-lysine in D_2O as a function of pD are shown in Figure 2. The protons adjacent to the α-NH_2 and ε-NH_2 groups experience a downfield shift of $0{\cdot}42$ and $0{\cdot}37$ p.p.m. respectively, on charging of the amino groups. These figures are appreciably less than those given above for charging amines in DCA or TFA and the difference is almost certainly related to the very different solvents used. The γ-CH_2 group undergoes a total downfield shift, due to charging of the two amino groups, of only $0{\cdot}10$ p.p.m. It is also seen that the centre of the S-shaped curve for the α-CH (pD $= 9{\cdot}6$) corresponds approximately with $pK'_2 \simeq 9{\cdot}8$ in D_2O, and the centre of the ε-CH_2 curve (pD $= 11{\cdot}1$) with $pK'_3 \simeq 11{\cdot}1$ in D_2O at 35°.[28]

Since placing a positive charge (by protonation) on the amide group results in a downfield chemical shift of the adjacent proton resonance, it is reasonable to

[23] Berger, A., Loewenstein, A., and Meiboom, S., *J. Am. chem. Soc.*, 1959, **81**, 62.
[24] Herbison-Evans, D., and Richards, R. E., *Trans. Faraday Soc.*, 1962, **58**, 845.
[25] Becker, B., and Davidson, A. W., *J. Am. chem. Soc.*, 1963, **85**, 159.
[26] Klotz, I. M., Russo, S. F., Hanlon, S., and Stake, M. A., *J. Am. chem. Soc.*, 1964, **86**, 4774.
[27] Stewart, W. E., Mandelkern, L., and Glick, R. E., *Biochemistry*, 1967, **6**, 150.
[28] Perrin, D. D., "Dissociation Constants of Organic Bases in Aqueous Solution." (Butterworths: London 1965.)

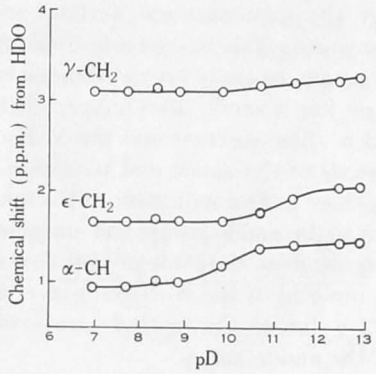


Fig. 2.—Chemical-shift data of the protons in L-lysine, as a function of pD in D_2O.

TABLE 1

CHEMICAL SHIFTS OF AMINES AND AMIDES IN $CDCl_3$, DCA, AND TFA

Chemical shift (τ) in p.p.m. Splitting of peaks in $CDCl_3$: s, singlet; b, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet

Material	Proton	$\tau(CDCl_3)$	$\tau(CDCl_3)$ $-\tau$(DCA)	$\tau(CDCl_3)$ $-\tau$(TFA)
N-Methylformamide	CH_3–N	d 7·20	0·20	0·33
	NH	b 6·71	4·36	4·50
N,*N*-Dimethylformamide	CH_3–N	s 7·03	0·22	0·33
	CH_3–N	s 7·13	0·27	0·31
N-Methylacetamide	CH_3–N	d 7·23	0·23	0·40 (0·44*)
	CH_3–CO	s 8·02	0·33	0·53 (0·50*)
	NH	b 2·81	0·94	1·49 (1·59*)
N,*N*-Dimethylacetamide	CH_3–N	s 6·98	0·18	0·32
	CH_3–N	s 7·07	0·27	0·42 (0·40*)
	CH_3–CO	s 7·93	0·30	0·50 (0·46*)
N,*N*-Dimethylacrylamide	CH_3–N	s 6·98	0·23	0·39
Diethyl acetaminomalonate	CH–N	d 4·75	0·23	0·32
$CH_3CONHCH(CO_2C_2H_5)_2$	CH_3–CO	s 7·93	0·21	0·28
	CH_3 (ester)	t 8·70	0·07	0·11
	CH_2 (ester)	q 5·73	0·13	0·21
	NH	b 3·35	1·08	1·47
N-Isopropylbenzamide	CH–N	m 5·72	0·00	0·25 (0·33†)
	CH_3	d 8·75	0·10	0·23
	NH	b 3·85	not obs.	0·83
Benzyloxycarbonyl-L-alanine	CH–N	m 5·67	0·20	0·21
$C_6H_5CH_2OCONHCH(CH_3)COOH$	CH_3	d 8·61	0·17	0·17
	CH_2–O	s 4·92	0·15	0·16
	C_6H_6	s 2·70	0·07	0·04
n-Propylamine	CH_2–N	t 7·37	0·55	0·48
	CH_3	t 9·10	0·13	0·12
	NH_2	b 8·92	5·89	6·20
n-Hexylamine	CH_2–N	t 7·31	0·57	0·53
	CH_3	t 9·10	0·02	0·02
	NH_2	b 8·95	5·88	5·77
N-Ethylaniline	CH_2–N	q 6·93	0·63	0·56
	CH_3	t 8·84	0·25	0·25
	NH	b 6·63	10·93	6·26

* Ref. 27. † $\tau(CDCl_3)-\tau(H_2SO_4)$.

assume that putting a negative charge (by deprotonation) on the amide should cause an upfield shift of this proton resonance. This is shown in Table 2. Triethylamine and piperidine are not strong enough bases to cause a chemical shift due to deprotonation, but sodium in benzene has a small effect. Apart from this, the n.m.r. spectra are essentially unchanged in these solvents and the N–H resonance is visible. However, when equimolar amounts of the amide and potassium t-butoxide are dissolved in dimethyl sulphoxide-d_6 there is (i) a reduction in the amount of the splitting of the proton resonance adjacent to the amide group, and an upfield chemical shift of 0·23 p.p.m. which is equal in magnitude to the average downfield shift of 0·24 p.p.m. on protonation in DCA, (ii) the removal of the N–H resonance, and (iii) the occurrence of a new resonance at 8·72 τ due to the methyl groups of t-butanol, which is produced by deprotonation of the amide group.

TABLE 2

CHEMICAL SHIFTS OF PROTONS (SHOWN IN BOLD TYPE) OF AMIDES IN $CDCl_3$ AND BASIC SOLVENTS

Material	$\tau(CDCl_3)-\tau$(solvent) in			
	Et_3N	Piperidine	Na/C_6H_6	Bu^tOK/DMSO-d_6
$HCONH-C\mathbf{H}_3$	0	[a]	−0·03	−0·22
$CH_3CONH-C\mathbf{H}_3$	0	[a]	−0·05	−0·23
$PhCONH-C\mathbf{H}(CH_3)_2$ [b]	0	−0·03	−0·07	−0·24

[a] Obscured by solvent.

[b] This amide is the most difficult one to protonate (see Table 1) due to a low pK value; conversely it is the easiest to deprotonate.

(ii) *Peptides*

The n.m.r. spectra of five protected peptides (see above) were determined in $CDCl_3$ and TFA. Interpretation of the α-CH resonances was made very difficult, and not improved appreciably by spectrum accumulation methods, because of the occurrence of (i) the glutamic ester CH_2 resonance on the upfield side of the α-CH resonance in all spectra except those of Z-Glu(OMe)-Glu(OMe)-Val-OPNB, (ii) a large amount of splitting of each α-CH resonance by adjacent groups combined with small chemical shifts between different α-CH groups in the molecule. Thus it was not possible to resolve separately the various α-CH resonances and a general downfield shift of 0·10 to 0·40 p.p.m. was noted on changing the solvent from $CDCl_3$ to TFA.

(iii) *Poly-L-amino Acids*

The downfield chemical shift of the α-CH proton in synthetic polypeptides in going from the α-helical form (in $CDCl_3$ or EDC) to the random-coil form (in DCA or TFA) is considered to be due to (i) the charging process shown in equation (1), and (ii) the collapse of the α-helix. The latter removes the particular orientation of the planar amide groups with respect to the α-CH protons and hence also randomizes the electric-field effect which the amide groups, by virtue of their permanent dipoles,

exert on the α-CH protons.[13,29] The two processes occur concurrently but it is assumed that it is possible to separate the contributions from each of them to the downfield chemical shift. The three methods used are summarized in Table 3 (cf. refs.[9,13,15,30] and Fig. 3).

TABLE 3

CHEMICAL-SHIFT DATA FOR POLY-AMINO ACIDS

Differences refer to downfield chemical shifts in p.p.m. Subscripts of τ signify: h, helix; uc, uncharged coil; cc, charged coil; u, uncharged; c, charged

Material	Solvent	Ref.	Proton	α-CH Protons			Side-chain
				$\tau_h - \tau_{uc}$	$\tau_{uc} - \tau_{cc}$	$\tau_h - \tau_{cc}$	$\tau_u - \tau_c$
Model amides	$CDCl_3$ to DCA	[a]	adj. to CONH	—	0·24[b]	—	—
Model amides	$CDCl_3$ to TFA	[a]	adj. to CONH	—	0·35[b]	—	—
Poly-γ-benzyl-L-glutamate	$CDCl_3$ to DCA	[a]	α-CH	—	—	0·64	—
	$CDCl_3$ to TFA	13	α-CH	—	—	0·58	—
		15	α-CH	—	—	0·55	—
Poly-DL-alanine	$CDCl_3$ to TFA	9	α-CH	—	0·20[c]	—	—
Poly-L-alanine	$CDCl_3$ to TFA	9	α-CH	0·44−0·20	—	0·45 | 0·44	—
		13	α-CH	= 0·24	—	0·42 |	—
Poly-L-methionine	$CDCl_3$ to TFA	13	α-CH	—	—	0·42	—
Poly-L-phenylalanine	$CDCl_3$ to TFA	30	α-CH	—	—	0·42	—
Poly-L-glutamic acid	D_2O	13	α-CH	0·10+0·04 = 0·14[d]	—	—	—
			β- and γ-CH_2 fused	—	—	—	−0·20
Poly-L-lysine	D_2O	Fig. 3	α-CH	0·22−0·02 = 0·20[e]	—	—	—
			ε-CH_2	—	—	—	0·37[f]
				Mean 0·19			

[a] This work.

[b] These represent mean values for downfield chemical shifts of protons in positions adjacent to amide groups resultant on transfer of model amides from $CDCl_3$ to DCA or TFA (see Table 1 and text).

[c] Extrapolated value (no result below 40% TFA).

[d] 0·10 is the mean value obtained from ref. 13 but there must be a small correction for the effect of the charged carboxylate group on the α-CH proton displaced four carbon atoms along the chain. A positive charge on nitrogen causes a downfield chemical shift of (1) 0·05 p.p.m. on the proton attached to the third carbon atom along the chain in lysine in D_2O (Fig. 2), (2) 0·12 p.p.m. on the proton attached to the third carbon atom along the chain in n-propylamine in $CDCl_3$–TFA, and (3) 0·02 p.p.m. on the proton attached to the sixth carbon atom along the chain in n-hexylamine in $CDCl_3$–TFA (Table 1). Assuming a negatively charged carboxylate group would cause an equal *upfield* chemical shift and noting that the effect in water is approximately half that in the organic solvent, a value of 0·04 is interpolated.

[e] The correction of the downfield chemical shift of the α-CH proton due to charging of the amino group attached to the fifth carbon atom along the chain in D_2O is interpolated as −0·02 p.p.m.

[f] This value is identical with that obtained for L-lysine (Fig. 2) and indicates that the ε-amino groups of poly-L-lysine are virtually 100% discharged before precipitation occurs. In this respect it differs considerably from poly-L-histidine which precipitates when only about one half of the imidazole rings are discharged (ref. 6).

In the first method, one measures in simple amides the downfield chemical shift (τ(uncharged)−τ(charged)) of protons adjacent to the amide group produced by charging and solvation of the latter. The second method is to measure the downfield chemical shift (τ(uncharged coil)−τ(charged coil)) of the α-CH proton due to charging and solvation of a *random coil* polypeptide (poly-DL-alanine) as compared

[29] Sternlicht, H., and Wilson, D., *Biochemistry*, 1967, **6**, 2881.

[30] Conti, F., and Liquori, A. M., *J. molec. biol.*, 1968, **33**, 953.

with the shift (τ(helix)—τ(charged coil)) of a closely similar polypeptide (poly-L-alanine) which also undergoes the helix to coil transition. Hence by subtraction (τ(helix)—τ(uncharged coil)) can be determined. The third method involves the calculation of the downfield chemical shift (τ(helix)—τ(uncharged coil)) during a helix to coil transition induced by charging of the side-chain rather than the peptide backbone, as occurs with poly-L-glutamic acid and poly-L-lysine in aqueous solution. In this method a small correction is made to the chemical shift for the effect of the charged side-chain on the α-CH proton (see Table 3).

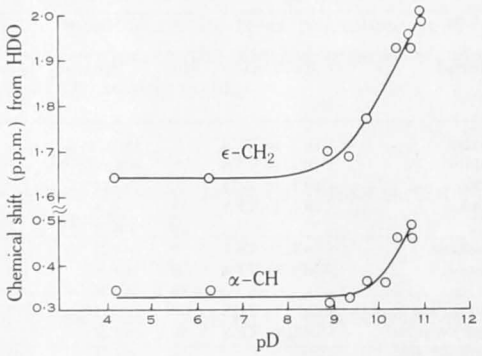


Fig. 3.—Chemical-shift data for poly-L-lysine in D_2O as a function of pD.

The downfield shift for the overall helix to coil transition (τ(helix)—τ(charged coil)) varies from 0·42 for poly-L-methionine to 0·64 for PBG. This variability is probably due mainly to the large variability of the downfield shift resulting from charging the amide group adjacent to the α-CH as shown in Table 3 and also found for simple amides (see Table 1). This is confirmed by the fact that the downfield shift of the α-CH proton due to collapse of the helix to an uncharged random coil is of the same order of magnitude for the three different polypeptides, particularly since the value for poly-L-glutamic acid is probably low due to precipitation before there is 100% conversion into the helical form. Although further confirmation is desirable it seems possible that (τ(helix)—τ(uncharged coil)) is a constant, independent of the polypeptide and the solvent, with an average value of 0·19 p.p.m.

(iv) *Poly-γ-benzyl-L-glutamate (PBG) in $CDCl_3$–DCA Mixtures*

The n.m.r. spectrum of high molecular weight PBG is not visible in trichloroethylene[7] and only the phenyl resonance can be observed in $CDCl_3$.[31] We have confirmed this behaviour which is due to association in $CDCl_3$.[32] When the aggregation but not the α-helical structure is removed by the use of dimethylformamide[2] the main n.m.r. resonances are still visible at high molecular weight.[33] However, by use of PBG of $DP_w < 53$, it is possible to observe the n.m.r. spectrum quite readily in $CDCl_3$ and in mixtures with DCA and TFA. A typical spectrum of PBG (DP_w 26) in $CDCl_3$ is shown in Figure 1 (curve *B*). One notes the considerable broadening of the various CH_2 resonances and apparent absence of the N–H resonance as compared with the spectrum in TFA, and also the downfield chemical shift of the α-CH reson-

[31] Marlborough, D. I., Orrell, K. G., and Rydon, H. N., *Chem. Commun.*, 1965, 518.
[32] Doty, P., Bradbury, J. H., and Holtzer, A. M., *J. Am. chem. Soc.*, 1956, **78**, 947.
[33] Bradbury, J. H., and Stubbs, G. J., *Nature*, 1968, **218**, 1049.

ance in TFA as compared with $CDCl_3$. The latter is in agreement with Markley *et al.*[13] and Bradbury *et al.*,[34] but not with Ferretti and Liquori (quoted in ref.[29]) who found no movement of the α-CH resonance on collapse of the helix.

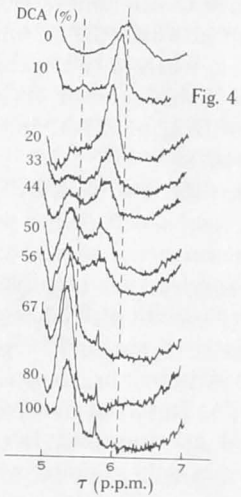


Fig. 4

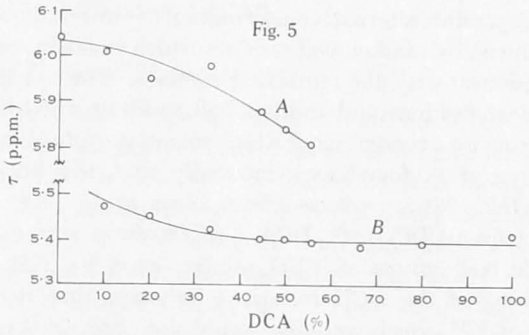
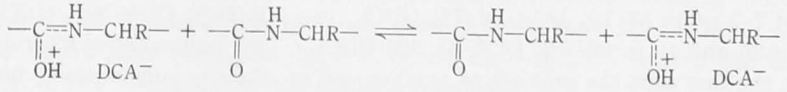


Fig. 5

Fig. 4.—Resonances due to α-CH, at 100 MHz, of PBG (DP_w 26), as a function of $CDCl_3$–DCA composition. The small, sharp resonance at 5·8 τ is identified, by its presence in pure DCA and its position, as due to the splitting of the proton resonance of DCA by ^{13}C.

Fig. 5.—Chemical-shift data for the helical (*A*), and random-coil (*B*), α-CH resonances of PBG (DP_w 26) in $CDCl_3$–DCA.

In Figure 4 is shown the occurrence of the two broad α-CH resonances obtained at 100 MHz for PBG in mixtures of $CDCl_3$ and DCA. This was obtained initially at 60 MHz[1,35] and is similar to the double α-CH resonances reported independently on other systems.[10,34] The single upfield α-CH resonance observed in 100% $CDCl_3$ is assigned to α-helical PBG and the downfield resonance obtained in 100% DCA to charged, random-coil PBG. In the transition region from helix to coil both these peaks are visible and their chemical shifts are recorded as a function of solvent composition in Figure 5. It is noted that the upfield, helical resonance moves downfield and broadens as the amount of DCA is increased and becomes only a very slight shoulder at 66·7% DCA. Similarly, the downfield, random-coil resonance moves upfield slightly and broadens as the amount of DCA is decreased. Both the chemical-shift behaviour shown in Figure 5 and the broadening of both peaks in the transition region is explained on the basis of proton exchange between the helical (uncharged) and random-coil (charged) forms of the amide groups as shown by equation (2).



(2)

[34] Bradbury, E. M., Crane-Robinson, C., Goldman, H., and Rattle, H. W. E., *Nature*, 1968, **217**, 812.

[35] Fenn, M. D., Thesis, Australian National University, 1967.

If this exchange did not occur or was very slow then one would obtain two α-CH resonances which maintained their positions and line-widths throughout the transition region. On the other hand, if the exchange is rapid then a single averaged resonance would be obtained which would move downfield as the amount of DCA was increased. Clearly, this case is an intermediate situation[36] in which the lifetime τ of each state is of the same order as $1/2\pi\delta \simeq 2{\cdot}5\times10^{-3}$ sec, where δ is the chemical shift (64 c/s) between the two states. An exchange rate of this order of magnitude is also found for low molecular weight PBG in mixtures of $CDCl_3$ and TFA,[15] although it has been proposed[34] that exchange is slow in this system.

A possible alternative explanation of our results is that the downfield α-CH peak is produced by random-coil residues which exchange only very slowly (hence possibly end-residues) with the remaining residues. The upfield resonance is then attributed to helical residues and random-coil residues which are exchanging rapidly, hence producing an average signal which moves downfield as the amount of DCA increases. However, it is found experimentally that the line-width of the upfield peak in PBG (DP_w 28) is narrowed from 22 c/s to 16 c/s at 60 MHz by the change of the solvent from $CDCl_3$–20% DCA to $CDCl_3$–20% deutero-DCA. In the latter solvent the peptide NH groups of PBG are replaced by ND and the observed line-width narrowing of the α-CH resonance indicates that it is spin–spin coupled with the adjacent NH. Such coupling would not occur if there were rapid proton exchange between helical and random-coil (charged) amide groups according to equation (2); hence this alternative explanation cannot be correct.

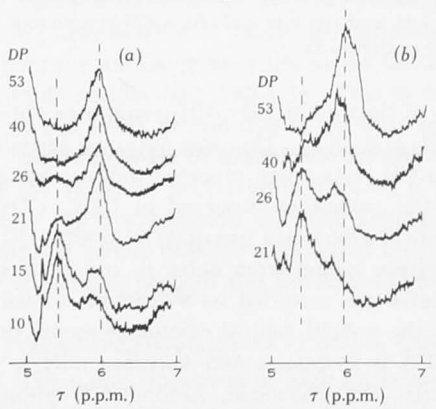


Fig. 6.—α-CH resonances at 100 MHz of PBG as a function of DP_w.
(*a*) 33·3% DCA–66·7% $CDCl_3$;
(*b*) 50% DCA–50% $CDCl_3$.

The effect of DP_w on the double α-CH resonances is shown in Figure 6 for two different solvent mixtures. Since the intensity of each resonance (area under the peak) is proportional to the number of protons involved, one can obtain an approximate estimate of the number of random-coil residues in the chain. It is found that this figure is substantially constant and independent of DP_w (below DP_w 53) with a mean value of $7{\cdot}3\pm2$ in 33·3% DCA–66·7% $CDCl_3$, 14 ± 2 in 50% DCA–50% $CDCl_3$, and between 26 and 40 in 66·7% DCA–33·3% $CDCl_3$. Two points are of interest here. Firstly, the fact that the number of random-coil residues is substantially independent of DP_w (below DP_w 53) might perhaps indicate that virtually all the random-

[36] Pople, J. A., Bernstein, W. G., and Schneider, H. J., "High Resolution Nuclear Magnetic Resonance." Ch. 10. (McGraw–Hill: New York 1959.)

coil residues are situated at the ends, since theories of helix–coil transitions predict that the amount of unwinding from the ends is independent of DP. However, it seems more likely that only the bulk of the random residues are at the ends, with a few internal breaks as depicted in Figure 7. Secondly, it was found in Part I from viscometric studies that as DP_w increases the transition from random coil to flexible helix occurs between DP_w 13 and 18 in 50% DCA–50% ethylene dichloride (EDC), and between DP_w 28 and 40 in 66·7% DCA–33·3% EDC. Since EDC and $CDCl_3$ can be considered as very similar non-interacting solvents for PBG, these latter results agree nicely with the figures of 14 ± 2 random-coil residues in 50% DCA–50% $CDCl_3$, and between 26 and 40 in 66·7% DCA–33·3% $CDCl_3$.

(*b*) *Proton 2 (N–H proton)*

The NH resonance in polypeptides is the most difficult to see of the various resonances[13,31] and to our knowledge has not yet been observed in a rigid, uncharged α-helical polypeptide. Thus, in PBG of low DP, it cannot be seen in $CDCl_3$ or dimethylformamide. However, it is readily observed in polypeptides in the "flexible helix" form shown in Figure 7 [7,9,10,13,27,34] although some workers[8,31] were only able to observe it in the random-coil form. The resonance is normally a broad doublet due to splitting by the adjacent CH group[9,13,27] but at 100 MHz two resonances characteristic of the helical and random-coil forms have been observed.[10,34] The broadening, which obscures the doublet even in the case of simple amides, is due to an intermediate rate of exchange of the NH proton with the solvent and the possibility of slight coupling with the ^{14}N nucleus.[15] In polypeptides in the helical form there is additional broadening due to the large correlation time of the molecule.[33]

As shown in Table 1 the downfield chemical shift of the NH resonance on protonation of simple amides is large and very variable. Similarly the change of the chemical shift resultant on the helix to coil transition varies for different polypeptides. Thus poly-L-alanine, poly-L-methionine, and PBG give a small upfield shift of 0·1–0·2 p.p.m., whilst poly-L-leucine and poly-β-benzyl-L-aspartate give a small upfield followed by a small downfield shift.[9,13] This complex behaviour is due to two factors: (1) Charging of the amide group causes a downfield shift which is very variable for simple amides and amounts to 0·2 p.p.m. for poly-DL-alanine.[9] (2) The hydrogen-bond disruption which accompanies the collapse of the helix results in an upfield shift.[13] The difficulty of observation of the NH resonance, coupled with the lack of predictive value of chemical-shift data, limits the usefulness of studies of this resonance.[14]

(*c*) *Proton 3 (COOH Proton of Acid)*

The carboxyl proton resonance consists of a singlet, which results from the rapid exchange of this proton between several structures including unmodified acid and various hydrogen-bonded forms of which one is shown (for DCA) in equation (1) and others have been postulated.[27] These authors have related the chemical shift τ_{obs} of this resonance by the standard equation

$$\tau_{obs} = \sum_i p_i \tau_i \qquad (3)$$

where τ_i is the chemical shift of the protons at the ith exchange site and p_i is the

mole fraction of exchanging protons at this site. Unfortunately, a complete analysis of the system is not possible because of the lack of information as to the identity of the various exchanging species and their respective chemical shifts and mole fractions.

Stewart *et al.*[9,27] have found that addition of TFA to a $CDCl_3$ solution of *N*-methylacetamide causes an initial large downfield chemical shift of the acid proton of TFA followed by a smaller upfield shift as TFA is added in excess of *N*-methylacetamide. This is interpreted in terms of charging of the amide with the formation of a 1 : 1 hydrogen-bonded complex with TFA. On the other hand, addition of TFA to a $CDCl_3$ solution of poly-L-alanine produces only a relatively small downfield chemical shift, from which they concluded that charging of the peptide groups of the polypeptide cannot occur to any appreciable extent.

The flaw in this argument is that *N*-methylacetamide was present at a molar concentration about 5·5 times as great as that of the peptide residues of poly-L-alanine. Thus at a particular concentration of TFA the mole fraction of protonated, hydrogen-bonded species with low values of τ is very much greater in the former than in the latter case, and hence by equation (3) the value of τ_{obs} is also much smaller in *N*-methylacetamide.

To check this point further, chemical-shift measurements were made in $CDCl_3$–DCA solutions of *N*-methylacetamide (0·44M) and PBG (0·46 moles peptide residues per litre, DP_w 21). The acid proton of DCA could not be observed in solutions containing 5% or less of DCA by volume, due presumably to exchange broadening and/or immobilization of the proton in structures of the type shown in equation (1). At 9% (v/v) the acid proton resonance in the solvent mixture alone and also that in the presence of PBG occurred at $-0{\cdot}50\ \tau$, whilst that in *N*-methylacetamide was 0·50 p.p.m. further downfield. This is attributed to an appreciable amount of protonation of *N*-methylacetamide, whereas the small amount of protonation of PBG would involve only a small fraction of the DCA molecules and cause no appreciable downfield chemical shift. At 20% DCA (v/v) the acid proton resonance in the presence of *N*-methylacetamide is 0·10 p.p.m. downfield from that in the presence of PBG or in the solvent alone, but at higher concentrations of DCA the chemical-shift difference becomes negligible by the operation of equation (3). It is concluded that chemical-shift measurements of the acid proton resonance (i) cannot be interpreted quantitatively, (ii) are very insensitive to protonation in dilute solution by virtue of equation (3), and (iii) do not provide evidence as previously supposed[9,27] to contradict the concept of charging of peptide groups.

(*d*) *Proton 4* (*α-CH proton of DCA*)

This proton resonance occurs as a singlet at $4{\cdot}00\ \tau$ and would be expected to move upfield as the proportion of the deprotonated form HCl_2CCOO^- (DCA^-) in the DCA increases, due to rapid exchange between DCA and DCA^-. Under normal conditions where $[DCA] \gg [DCA^-]$, the upfield shift will be very small; hence it is only useful to work in solutions in which [DCA] is small. Accordingly, measurements of this resonance were made in $CDCl_3$ solutions containing 1–9% by volume of DCA in the presence and absence of PBG (10% w/v, DP_w 21). Addition of PBG caused a maximum upfield shift of 0·05 p.p.m. in 1% DCA solution. If one assumes that the total upfield shift from DCA to DCA^- is 0·50 p.p.m., then a simple calcu-

lation shows that there is about 2% charging of the peptide groups of PBG in 1% DCA–99% $CDCl_3$. This figure is less than the estimate made by Hanlon.[5]

(e) *Charging of Amide Groups of Poly-L-Amino Acids in Mixtures of EDC or $CHCl_3$ with DCA and TFA*

Because of the apparent conflict of opinion (see Introduction) on the question of charging of the amide groups of poly-L-amino acids in mixtures of non-interacting solvents ($CHCl_3$, EDC) and strong organic acids (DCA and TFA) it is proposed to summarize the present situation.

(i) *Infrared Spectroscopy*

Klotz and co-workers[3–5] were the first to propose the charging of the amide group in polypeptides on the basis of the appearance of an infrared band at about 1·51 μ in the presence of DCA and TFA. However, the extent of charging of the amide group of high molecular weight PBG in EDC–DCA mixtures in the region 2–70% DCA is purported to be about 20% rising to 60%.[5] This figure is greatly in excess of the amount obtained by other methods.

(ii) *Dielectric Constant, Electric Birefringence, and Conductivity*

Watanabe and co-workers[37,38] measured these quantities for PBG solutions in EDC. On addition of a small amount of DCA there was a marked decrease in the dielectric constant and the electric birefringence, and an increase in the specific-conductance increment of the solution. This is explained in terms of protonation of the carboxyl terminal end of the long helical polymer, but the flexible helix structure shown in Figure 7 is probably a reasonable alternative explanation of their results.

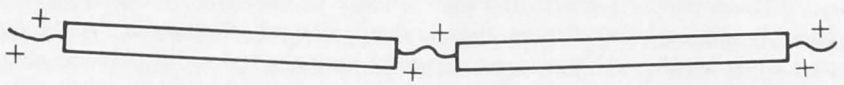


Fig. 7.—Schematic representation of rigid, helical rod with short, charged, random-coil breaks. This is called the flexible helix structure, as shown in Part I, Figure 9, structure 2.

(iii) *Viscosity*

The occurrence of a polyelectrolyte effect for poly-L-amino acids, in DCA and even in 80% EDC–20% DCA solutions of PBG of $DP_w \gtrsim 155$, is considered to be unequivocal *proof* of charging of the polypeptide.[2] However, the exponent in the Mark–Houwink equation decreases from 1·65 in the absence of DCA to 1·39 in 66·7% DCA, thus showing that the flexibility of the helix increases with increasing amount of DCA, but that the helical structure is still maintained up to the transition point.[39] The total amount of charging on the flexible helix structure is therefore likely to be small.

(iv) *Nuclear Magnetic Resonance*

It has been the major thesis of this paper to measure the downfield chemical shift of the α-CH proton of simple amides resulting from protonation (and the

[37] Watanabe, H., and Yoshioka, K., *Biopolymers*, 1966, **4**, 43.
[38] Watanabe, H., Yoshioka, K., and Wada, A., *Biopolymers*, 1964, **2**, 91.
[39] Doty, P., and Yang, J. T., *J. Am. chem. Soc.*, 1956, **78**, 498.

equivalent upfield shift produced by deprotonation) and to relate this with the downfield shift produced in polypeptides during the helix to coil transition. The latter shift can be divided into two parts, one due to the charging process and another to the transition from a helical form to an uncharged random coil. The whole forms a consistent scheme which gives strong support to the idea of charging. Stewart *et al.*[9,27] have obtained chemical-shift data which purport to show the absence of charging, but we believe that their results were interpreted incorrectly, and have carried through further experiments to confirm our assessment (see above). Ferretti[10] finds that neither the NH line-widths nor the chemical shifts of the helical and random-coil forms alter as TFA is added to a solution of the polypeptide in $CDCl_3$, and he cites this as evidence for lack of protonation. We would not agree with this conclusion, as it seems likely that the large line-widths are produced largely by exchange broadening in which the rate of exchange may be independent of TFA concentration and bear no direct relation to protonation. Also, the chemical shifts of the α-CH resonances of the random-coil and helical forms of PBG are, like the corresponding NH resonances, only slightly dependent on TFA concentration, yet this does not give any evidence for or against protonation.

(v) *Circular Dichroism and Optical Rotatory Dispersion*

Quadrifoglio and Urry[11] have measured the circular dichroism of high molecular weight PBG and poly-γ-methyl-L-glutamate in non-interacting solvents to which TFA or DCA is added. They find that no change occurs in the ellipticity at 222 mμ until the normal helix to coil transition point is reached. Similarly, on the addition of TFA to a solution of PBG (Pilot sample) in EDC, there is no appreciable change in the optical rotatory dispersion around 235 mμ until the helix to coil transition occurs.[12] These results seem to indicate absence of charging of the flexible helix, but Quadrifoglio and Urry[11] note that a small amount of charging would not be detected by this method. Since our viscocity and n.m.r. results indicate charging of about 10–20 residues of the flexible helix at low molecular weights with (probably) a slow increase with increase of molecular weight, it is quite probable that the amount of charging of a high polymer in the flexible helix form would be only several per cent; much less than predicted.[5] However, if this explanation is correct, then examination of a sample of PBG of low molecular weight by c.d. and o.r.d. should produce an appreciable effect.

At the present time there is an overwhelming amount of evidence from viscosity, infrared, dielectric constant, electric birefringence, and conductivity studies to support the concept of charging of amide groups of polypeptides in mixtures of non-interacting and strong acid solvents. N.m.r., c.d., and o.r.d. are less sensitive indicators of small amounts of charging and hence studies made on polypeptides of low molecular weight (where there is a greater fractional degree of charging) should be extended from n.m.r. to the other two methods.

(*f*) *Flexible Helix Model*

The flexible helix model depicted in Figure 7 consists of helical segments separated by short, charged, random-coil breaks at both ends and in the interior of the molecule. Various features of this model will now be elaborated.

(i) It is assumed that a charged amide group is not involved in the normal intramolecular hydrogen bonding of the α-helix and is thus designated as a random-coil residue. However, there is the possibility of the occurrence of modified types of hydrogen-bond arrangements which involve charged groups.[5]

(ii) The rate constant for exchange of protons between uncharged (helical) and charged (random-coil) residues of PBG of low DP_w is considered to be of the order of 400 sec^{-1} in $CDCl_3$–DCA and $CDCl_3$–TFA solutions. Various estimates have been made for different polypeptides[9,10,13,27,34] and these will be discussed in detail elsewhere.[15]

(iii) The addition of increasing amounts of DCA to a solution of PBG in EDC (or $CHCl_3$) causes increasing charging of the amide groups until the electrostatic repulsion is sufficient to cause the complete disruption of the flexible helix at the flexible helix to coil transition point (about 75% DCA).[39]

(iv) Both the n.m.r. and viscosity results (non-occurrence of polyelectrolyte effect at $DP_w > 155$ in 20% EDC–80% EDC) indicate a decrease in the fraction of charged, random-coil residues with increase of DP_w. This is consistent with theories of helix to coil transitions which predict that the amount of unwinding of residues from the ends of the helix is independent of DP_w.[40]

(v) The flexibility of the flexible helix is shown by the occurrence of the polyelectrolyte effect in 20% DCA–80% EDC, and the magnitude of the exponent $\alpha \simeq 1{\cdot}4$ in the Mark–Houwink equation. The latter also shows that the structure is still rod-like, in confirmation of the early work of Doty and co-workers (summarized in Part I), rather than a random-coil structure.

(vi) The occurrence of a polyelectrolyte effect in DCA for poly-β-benzyl-L-aspartate, poly-ε-benzoxycarbonyl-L-lysine (Part I), and poly-L-methionine in TFA,[41] and the large downfield shift of the α-CH resonance in the helix to coil transition of poly-L-alanine, poly-L-phenylalanine, and poly-L-methionine (Table 3) show that charging of the amide group occurs quite generally with polypeptides in DCA and TFA. The flexible helix structure is therefore proposed as a general structure for those polypeptides which are soluble in organic solvents and undergo the helix to coil transition.

Acknowledgments

We wish to thank Professor Paul Doty for a gift of the residual samples of PBG from earlier studies by the Harvard group, and Dr F. H. C. Stewart for samples of small model peptides. We thank Mr A. Arandjelovic for the spectra obtained with the Varian HA100 instrument and the Australian Research Grants Committee for the provision of funds for the purchase of the PDP8–S computer.

[40] Birshtein, T. M., and Ptitsyn, O. B., "Conformations of Macromolecules." (Interscience: New York 1966.)

[41] Bradbury, J. H., and Chapman, B. E., unpublished data.

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HELIX TO COIL TRANSITION IN POLY-L-AMINO ACIDS

III.* KINETIC SCHEME FOR POLY-γ-BENZYL-L-GLUTAMATE

By J. H. Bradbury,† M. D. Fenn,† and A. G. Moritz‡

[Manuscript received July 21, 1969]

Summary

Double resonance experiments show that the α-CH proton magnetic resonance in poly-γ-benzyl-L-glutamate (PBLG) is not coupled to the ^{14}N nucleus but is coupled to the NH and β-CH_2 protons. The breadth of the resonance in the random coil form of the polypeptide is due to this coupling and is independent of degree of polymerization.

The coupling of NH and α-CH protons and the occurrence of NH proton resonances are indicative of slow exchange of NH protons with solvent. In addition, the occurrence of two α-CH resonances indicates slow exchange between the two-proton magnetic states. However, the rate of the helix to coil transition in PBLG obtained by other workers is about 10^4 times as fast as these exchange processes.

The experimental data are rationalized by a kinetic scheme which envisages a *rapid* equilibrium between helical and coil residues, and a *slow* equilibrium involving protonation of the peptide groups by the strong organic acid. The upfield α-CH resonance is attributed to the uncharged helical and coil residues and the downfield resonance to the charged helical (if present) and charged coil residues.

Introduction

In the previous papers of this series we have produced evidence from viscometry[1] and nuclear magnetic resonance spectroscopy[2] of poly-L-amino acids to support very strongly the postulate[3–5] of charging of the peptide groups in the presence of appreciable amounts of the strong organic acids dichloroacetic acid (DCA) and trifluoroacetic acid (TFA). Nevertheless there has been considerable controversy over the question of charging and the conflicting evidence has been summarized.[2] Since Part II[2] was written further evidence which purports to show the absence of charging has been obtained from circular dichroism studies on model amides[6] and infrared studies on polyalanine.[7] On the other hand, the observation that the addition of amines, water,

* Part II, *Aust. J. Chem.*, 1969, **22**, 357.

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[1] Bradbury, J. H., and Fenn, M. D., *J. molec. Biol.*, 1968, **36**, 231.
[2] Bradbury, J. H., and Fenn, M. D., *Aust. J. Chem.*, 1969, **22**, 357.
[3] Hanlon, S., Russo, S. F., and Klotz, I. M., *J. Am. chem. Soc.*, 1963, **85**, 2024.
[4] Hanlon, S., and Klotz, I. M., *Biochemistry*, 1965, **4**, 37.
[5] Hanlon, S., *Biochemistry*, 1966, **5**, 2049.
[6] Bovey, F. A., *Pure appl. Chem.*, 1968, **16**, 417.
[7] Bradbury, E. M., and Rattle, H. W. E., *Polymer*, 1968, **9**, 201.

etc.[8] and particularly dichloroacetate ion[9] causes the coil to helix transition in poly-L-amino acids (see mechanism below), is further evidence in favour of charging. We believe that the unequivocal *proof* of charging of the peptide group stems from the fact that normally uncharged poly-L-amino acids behave as polyelectrolytes in DCA and TFA.[1]

A second related question concerns the rate of the helix to coil transition. It has been shown that the relaxation time for the helix to coil transition of polypeptides of molecular weight 20000–380000 is $< 10^{-5}$ sec in poly-L-glutamic acid,[10] $< 2\times10^{-6}$ sec in poly-L-tyrosine,[11] $1\cdot7\times10^{-8}$ sec in poly-L-ornithine,[12] and 5×10^{-7} sec in poly-γ-benzyl-L-glutamate (PBLG) at the centre of the transition.[13] For polypeptides of degree of polymerization (DP) $>$ about 100 (molecular weight $>$ about 10000) the n.m.r. resonance of the α-CH proton is normally too broad to observe in the helical form, but during the transition to the random coil occurs as a broad single peak[14–16] (with one exception[17]) which moves downfield.[2] This has been interpreted as indicative of a magnetic environment for the α-CH protons which is the average of that due to the helical and random coil residues, which are in rapid equilibrium with a relaxation time $\leqslant 2\text{–}6\times10^{-3}$ sec.[2,15] On the other hand with polypeptides of $DP < 100$ there have been many reports of the occurrence of double α-CH resonances in the helix-coil transition region since the initial observation of this effect.[18–20] Double peaks are also sometimes observed for the NH protons.[16–18,21] It is generally assumed that the upfield α-CH peak represents the protons attached to helical residues and the downfield peak represents α-CH protons attached to random coil residues.[2,16,18] The relaxation time for exchange between helical and random coil residues would thus be much larger than that for polypeptides of high molecular weight and various estimates have been given with a minimum of about $2\cdot5\times10^{-3}$ sec.[2,18]

An explanation is required for the "apparent" increase in the rate of the helix to coil transition for polypeptides with increase of DP as shown by n.m.r. measurements. However, of greater significance is the large discrepancy (by a factor of 10^4) between the relaxation times for the helix to coil transition as determined by dielectric relaxation[13] and by n.m.r. It is the object of this paper to propose a kinetic scheme

[8] Steigman, J., Verdini, A. S., Montagner, C., and Strasorier, L., *J. Am. chem. Soc.*, 1969, **91**, 1829.

[9] Zezine, A. B., Bakeiev, N. F., Gourevitch, V. M., and Kozlov, P. V., IUPAC International Symposium on Macromolecular Chemistry, Toronto, Canada, 1968, B2–13.

[10] Lumry, R., Legare, R., and Miller, W. G., *Biopolymers*, 1964, **2**, 489.

[11] Hamori, E., and Scheraga, H. A., *J. phys. Chem.*, 1967, **71**, 4147.

[12] Hammes, G. G., and Roberts, P. B., *J. Am. chem. Soc.*, 1969, **91**, 1812.

[13] Schwarz, G., and Seelig, J., *Biopolymers*, 1968, **6**, 1263.

[14] Stewart, W. E., Mandelkern, L., and Glick, R. E., *Biochemistry*, 1967, **6**, 143.

[15] Markley, J. L., Meadows, D. H., and Jardetzky, O., *J. molec. Biol.*, 1967, **27**, 25.

[16] Bradbury, E. M., Crane-Robinson, C., Goldman, H., and Rattle, H. W. E., *Nature*, 1968, **217**, 812.

[17] Ferretti, J. A., and Paolillo, L., *Biopolymers*, 1969, **7**, 155.

[18] Ferretti, J. A., *Chem. Commun.*, 1967, 1030.

[19] Fenn, M. D., Thesis, Australian National University, 1967.

[20] Bradbury, J. H., and Fenn, M. D. in "Symposium on Fibrous Proteins, Australia 1967." (Ed. W. G. Crewther.) p. 69. (Butterworths: Sydney 1968.)

[21] Haylock, J. C., and Rydon, H. N., in "Peptides." (Ed. E. Bricas.) p. 19. (North Holland: Amsterdam 1968.)

for the helix to coil transition which is consistent with the rate measurements and the n.m.r. results. An examination of the spin–spin coupling between α-CH protons and adjacent nuclei has also been useful in the development of the theory.

Experimental

The preparation and properties of PBLG samples is given elsewhere[1] and also the sources and methods of purification of n.m.r. solvents.[1,2] Deuterotrifluoroacetic acid (TFA-*d*), spectroscopic grade, was obtained from E. Merck. All n.m.r. spectra were obtained using PBLG concentrations of 10% w/v with a Varian HA100 spectrometer or else a Varian HA60 spectrometer, modified to allow the performance of double resonance experiments.[22] Line widths represent the mean of not less than three scans and chemical shifts are recorded on the τ scale using an internal standard of tetramethylsilane.

Results

Figure 1(*a*) shows the change in the α-CH resonances during the helix to coil transition in $CDCl_3$–TFA; the results are very similar to those given elsewhere.[16]

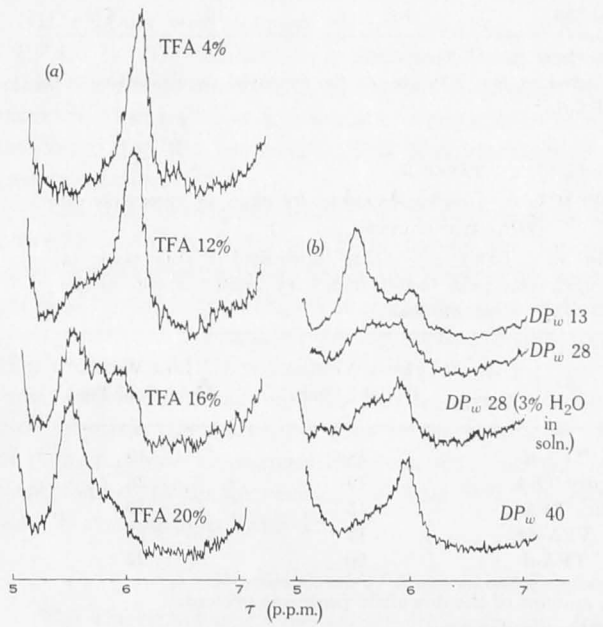

Fig. 1.—α-CH resonances at 100 MHz of (*a*) PBLG (DP_w 26) in $CDCl_3$ containing varying amounts of dry TFA. (*b*) PBLG of varying DP_w in 85% $CDCl_3$–15% TFA-*d*.

Figure 1(*b*) shows the effect of *DP* on the double α-CH resonance at a solvent composition near the centre of the transition. It is noted that as *DP* increases the area of the upfield resonance is increased with respect to the downfield peak as has already been observed for the case of the $CDCl_3$–DCA solvent system.[2] The results of the line width measurements made at 60 MHz with spin decoupling and at 100 MHz are given in Tables 1 and 2 respectively.

[22] Long, G. J., and Moritz, A. G., *Molec. Phys.*, 1968, **15**, 439.

In order to check on the prediction[23] that the α-CH resonance should have a different chemical shift in a right-handed helix (PBLG) from that of a left-handed helix (poly-γ-benzyl-D-glutamate, PBDG) we prepared a sample of the latter of

TABLE 1

SPIN DECOUPLING AT 60 MHz OF PROTON RESONANCES OF PBLG

Solvent	Sample of PBLG[a]	Spin Decoupling Experiment	Line Width (Hz) α-CH	Line Width (Hz) Benzyl CH_2
$CDCl_3$	PBLG	nil	30	10
$CDCl_3$	PBLG-*d*[b]	nil	22	10
TFA	PBLG	nil	22	5·0
TFA	PBLG	irrad. of ^{14}N	22	5·0
TFA-*d*	PBLG-*d*[b]	nil	16	5·0
TFA-*d*	PBLG-*d*[b]	irrad. of β- and γ-CH_2 groups at τ 7·75	5·3	5·0
TFA	PBLG, DP_w 295	nil	22	5
TFA-*d*	PBLG, DP_w 233	nil	16	4·5

[a] $DP_2 = 21$, except where stated otherwise.

[b] PBLG was deuterated at the NH groups by repeated precipitation with D_2O from a solution in TFA-*d*.

TABLE 2

LINE WIDTH (Hz) AT 100 MHz OF α-CH RESONANCES OF PBLG AS FUNCTION OF DP_w AND SOLVENT

Upfield PBLG peak in 95% $CDCl_3$–5% acid; downfield PBLG peak in 80% $CDCl_3$–20% acid (v/v). One peak visible with very small amounts of the second peak

DP_w of PBLG	Acid	Line Width Upfield Peak	Line Width Downfield Peak
13	TFA-*d*	28[a]	19
26	dry TFA	19	28
28	dry TFA-*d*	15	19
28	TFA-*d*[b]	17	37
40	TFA-*d*	20	25

[a] An appreciable amount of the downfield peak was present.

[b] Water (3%) in the mixed solvent.

DP_n (determined by n.m.r. end group measurements[2]) of 17±2. The chemical shift of the α-CH resonance in both the left-handed helical form (in $CDCl_3$) and in the random coil form (in TFA) agreed with that of PBLG in these solvents and confirms the results of E. M. Bradbury *et al.*[24]

[23] Sternlicht, H., and Wilson, D., *Biochemistry*, 1967, **6**, 2881.

[24] Bradbury, E. M., Carpenter, B. G., Crane-Robinson, C., and Rattle, H. W. E., *Nature*, 1968, **220**, 69.

Discussion

(a) *Spin–Spin Coupling of α-CH Proton*

The α-CH proton has the possibility of being spin–spin coupled to the adjacent ^{14}N nucleus, the NH proton, and the β-CH_2 protons of the side-chain. The double resonance experiment reported in Table 1, in which the ^{14}N nucleus was irradiated and there was no reduction in the line width of the α-CH resonance, shows that there is no coupling between the ^{14}N nucleus and the α-CH proton. This effect is well known and is probably due to the rapid relaxation of the ^{14}N nucleus between its three spin states, which allows decoupling of the ^{14}N nucleus from the protons adjacent to it (NH and α-CH).[14,25,26]

On the other hand there is a reduction of the α-CH line width after replacement of the NH by ND either by use of deutero-PBLG (for n.m.r. measurements in $CDCl_3$) or simply by measurements made in TFA-*d* or DCA-*d*.[2] The sharpening of the α-CH proton resonance produced by replacement of NH by ND therefore occurs with both the upfield and downfield peaks (see also Table 2). It must be due to coupling of NH and α-CH protons, since there is no change in the line width of the benzyl CH_2 protons. This coupling, also observed by Bovey,[6] indicates a rate of exchange of NH protons with solvent (or one another) which is relatively slow (order of $< 10^3$ sec^{-1}). This conclusion is also implicit in the widespread observation under certain conditions of solvent, etc. of NH resonances in simple amides, polypeptides (summarized in ref.[2]), and in proteins. The rate of exchange is of the same order as that found for the protolysis of *N*-methylacetamide and related compounds in aqueous solutions.[27–29]

The final decoupling of the α-CH proton from the β-CH_2 protons, achieved by irradiation of the latter in TFA-*d*, caused the line width to fall to a value which is only slightly greater than that of the benzyl-CH_2 protons (see Table 1). It is thus clear that the breadth of the α-CH resonance over and above that of other resonances in polypeptides in the random coil form is due to spin–spin coupling with the adjacent NH proton and β-CH_2 protons. In the helical form of the polypeptide there is, of course, additional broadening due to the greater rigidity of the rod-like structure, which increases the correlation time of the protons.[25] The line width of the benzyl and phenyl proton resonances increases with increase of the *DP* of the rod, but is independent of *DP* in the random coil form.[30,31] The latter effect is now recorded for the α-CH resonance in Table 1.

(b) *Summary of Experimental Information*

The kinetic scheme must satisfactorily account for the following experimental results:

[25] Emsley, J. W., Feeney, J., and Sutcliffe, L. H., "High Resolution Nuclear Magnetic Resonance Spectroscopy." Vol. 2. (Pergamon: Oxford 1968.)
[26] Bovey, F. A., and Tiers, G. V. D., *J. Polym. Sci.* A-1, 1963, 849.
[27] Berger, A., Loewenstein, A., and Meiboom, S., *J. Am. chem. Soc.*, 1959, **81**, 62.
[28] Takeda, M., and Stejskal, E. O., *J. Am. chem. Soc.*, 1960, **82**, 25.
[29] Schleich, T., Gentzler, R., and Hippel, P. H. von, *J. Am. chem. Soc.*, 1968, **90**, 5954.
[30] Bradbury, J. H., and Stubbs, G. J., *Nature*, 1968, **218**, 1049.
[31] Chapman, B. E., Thesis, Australian National University, 1968.

(1) As shown in Section (*a*) the rate of exchange of NH protons with the solvent (or with other similar protons in the polypeptide chain, which is likely to be a much slower process[27]) is such that $k < 10^3$ sec^{-1}, both for the helical and randomly coiled polypeptide at low and high molecular weight.

(2) At $DP < 100$ there are two α-CH proton resonances[16–20] which indicate two distinct types of α-CH protons having different magnetic environments and appreciable lifetimes (order of $> 10^{-3}$ sec). At higher values of *DP* only one α-CH resonance is normally observed and it has been proposed[16] that this could be due to more rapid exchange between the two states as *DP* increases. This is probably incorrect since there is recent evidence for two α-CH resonances in poly-L-methionine[21] and in poly-L-alanine of high *DP*. A simpler explanation is that progressive broadening of the upfield α-CH resonance occurs with increase of *DP*, which causes fusion of the resonances.

(3) Kinetic studies[10–13] show that the rate of the helix to coil transition in polypeptides of high *DP* is very large indeed; the relaxation time for PBLG is 5×10^{-7} sec at the centre of the transition.[13]

It is clear that any kinetic scheme must allow for relatively slow processes for protonation of the peptide group and for the transformation between the two states of the α-CH resonance together with a fast process for the helix to coil transition.

(*c*) *Proposed Kinetic Scheme*

In this scheme there are two possible alternative paths represented by equations (1) and (2) or (3) and (4), for the conversion from a helical residue h with intramolecular hydrogen bonds intact to a protonated random coil residue cH^+:

$$h \rightleftharpoons c \qquad \text{fast} \qquad (1)$$

$$c + TFA \rightleftharpoons cH^+ + TFA^- \qquad \text{slow} \qquad (2)$$

$$h + TFA \rightleftharpoons hH^+ + TFA^- \qquad \text{slow} \qquad (3)$$

$$hH^+ \rightleftharpoons cH^+ \qquad \text{fast} \qquad (4)$$

In this scheme c represents a random coil residue which will be involved in hydrogen bonding with the solvent (designated as TFA but it could equally well be DCA or even formic acid[32]) and hH^+ refers to a helical residue which is also protonated. The latter cannot readily be accommodated in the helix and would form highly distorted hydrogen bonds[5]; and it therefore seems likely that reactions (3) and (4) only occur to a small extent compared with reactions (1) and (2). For the purposes of this discussion it does not matter whether the structure of the charged random coil residue cH^+ is written with the proton attached on the nitrogen or the oxygen.[27–29] Other processes are obviously possible, such as direct proton exchange between charged and uncharged peptide groups as proposed in equation (2) of Part II.[2] Because the rate of this process for simple amides is shown to be much slower than exchange with solvent it is not considered further here, although in view of the stacking of the peptide groups in the helix it could possibly contribute to the slow rate processes (equations (2) and (3)).

[32] Lotan, N., Bixon, M., and Berger, A., *Biopolymers*, 1967, **5**, 69.

(*d*) *Explanation of N.M.R. Results*

It is clear that the above kinetic scheme satisfies the two requirements of (1) a very rapid transition between helix and coil and (2) a much slower process for the protonation of the peptide groups and exchange of the NH proton with solvent. The double α-CH resonance then consists of an upfield peak which is a composite of h and c residues (in rapid equilibrium) and the downfield peak which is composed of hH^+ and cH^+ residues in rapid equilibrium (concentration of hH^+ is probably very small).

As shown in Table 3 of ref.[2] the downfield chemical shift ($\tau_h - \tau_c$) resultant on the transition from helix to coil h → c, was calculated by three different methods to be about 0·19 p.p.m. However, this may be an overestimate at least for the case of PBLG, since it has been shown that the α-CH resonance of the random coil form of poly-γ-benzyl-DL-glutamate has the same chemical shift in $CDCl_3$ at 100° as has helical PBLG.[24] Also the observation (see above and ref.[24]) of the same chemical shift for the α-CH resonance of right-handed, helical PBLG and left-handed, helical PBLG indicates that there is little or no chemical shift of the α-CH resonance which can be attributed to the special orientation of the anisotropic peptide groups in the helix. It is thus likely that ($\tau_h - \tau_c$) for PBLG is $< 0{\cdot}19$ p.p.m. The upfield α-CH resonance would then be a single peak which gradually moved downfield as more acid was added. Similarly, the downfield α-CH resonance might be expected to move downfield providing that there is an appreciable amount of hH^+ produced by equation (3). Both these downfield shifts have been observed experimentally (see Fig. 5, ref.[2]), although they can also be interpreted in another way which is equally valid.[2]

According to previous ideas[2,16,18] the upfield α-CH resonance represented helical (h) residues and the downfield resonance random coil residues ($c+cH^+$). The relative areas of these peaks have been used to calculate the percentage helix which in one case agreed[16] and in another disagreed[21] with the value obtained from optical rotatory dispersion measurements. We calculated the number of random coil residues,[2] which agreed approximately with an estimate obtained from viscometry.[1] On the basis of the new scheme the upfield α-CH resonance is due to ($h+c$) residues and the downfield resonance is produced by ($cH^+ + hH^+$) residues. Thus for the particular case in which the intermediates in equations (1)–(4), viz. hH^+ and c, are present in low concentration compared with h and cH^+,* the new scheme approximates to the previous one.

The addition of water to PBLG in $CDCl_3$–TFA mixtures has the effect of greatly broadening the downfield resonance whilst not affecting appreciably the line width of the upfield peak as shown in Table 2. This effect is explained by the formation of TFA^- by the reaction

$$H_2O + TFA \rightleftharpoons H_3O^+ + TFA^-$$

which shifts the equilibrium in reactions (3), (2), and (1) with formation of increased amounts of c and h as shown in Figure 2. The increased concentration of TFA^- also increases the rate of the reverse reactions (2) and (3) sufficiently to cause partial collapse of the resonance due to cH^+ and hH^+. In the case of the upfield peak in

* $[hH^+]$ is probably small (see above) and [c] *may be* $< [cH^+]$, since $[TFA] \gg [TFA^-]$ in reaction (2), but the actual situation is dependent on the equilibrium constant for this reaction.

95% $CDCl_3$–5% TFA there is very little of the downfield peak present and addition of water reduces its amount still further, but (as expected) does not affect the breadth of the peak.

(e) *Various Possible Magnetic States of α-CH Protons*

It is important to recognize that there are, in principle, more magnetic states of the α-CH proton than those given in the proposed scheme above. This is because the magnetic state of the α-CH proton is dependent on the state of both peptide groups adjacent to it. For example, it would be anticipated that the α-CH resonance in structure (I) would occur downfield from that in the structure (II).

(I) (cH⁺cH⁺) (II) (cH⁺c)

If there is rapid equilibrium between helical and random residues, as postulated above, then this would produce three averaged resonances for the α-CH proton, viz. an upfield peak due to hh, cc, etc. states, a midfield peak due to hcH^+, ccH^+ etc., states, and a downfield peak due to cH^+cH^+, hH^+cH^+ etc. states.* Such triple resonances have not been observed (except for one case in which a small midfield peak was assigned to end residues[16]) for the obvious reason that the α-CH resonances are so broad. Thus, as shown in Table 3 of Part II, $\tau(h)-\tau(cH^+)$ varies from 0·42 to 0·64 p.p.m., depending on the particular polypeptide, whereas the line width of resonances is usually $\simeq 20$ Hz (see Tables 1 and 2) which equals 0·33 p.p.m. at 60 MHz and 0·20 p.p.m. at 100 MHz. The likelihood of observing three such resonances in a space of from 0·42 to 0·64 p.p.m. is therefore small even at 100 MHz. Published spectra at 220 MHz show the two α-CH resonances of poly-L-methionine with line widths of 0·12–0·16 p.p.m.[21] and the normal[2,15] amount of broadening in the helix-coil transition region. Perhaps this broadening is at least partly caused by appreciable concentrations of protons in intermediate magnetic states.

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* The need for the consideration of two residues is analogous to the analysis of tacticity of synthetic polymers where the term diad has been used to describe two residues.[33]

[33] Bovey, F. A., *Accounts Chem. Res.*, 1968, **1**, 175.